The Regulation and Function of 11β-Hydroxysteroid Dehydrogenase (11β-HSD1) in Pancreatic β-cells

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DECLARATION

I hereby declare that this thesis was composed by me and the data presented represent my own work, with the exceptions listed below:

The primers for 11β-HSD1 promoters were generously provided by Prof. Karen Chapman. Cardiovascular Science, QMRI, University of Edinburgh, UK.

The mouse insulin-I promoter was kindly gift from Dr. Mark Magnusson, Vanderbilt University, USA.

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The radioimmunoassy measurement for insulin content was performed by Sophie Turban. Molecular Metabolism Group, QMRI, University of Edinburgh, UK.

I declare that this work has not been submitted for any other professional qualification or degree.

Xiaoxia Liu
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DEDICATION

To the people thousands of years after who want to know me and this period history!

List of Manucripts and Meeting Abstract

Manuscripts

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Abstracts

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Presentation

Xiaoxia Liu, Sophie Turban-Rajaonah, Lynne Ramage, John Mullins, Jonathan R Seckl, Nicholas M. Morton (2009). Dose-Dependent Effects of Pancreatic β-cell-specific 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) Overexpression on Insulin Secretion. 4th Integrative Physiology Postgraduate Conference, University of Aberdeen, Aberdeen, UK.

ABSTRACT

Diabetes Mellitus is characterized by high blood sugar and is caused by resistance to (type 2) or insufficiency of (type 1) the pancreatic β-cell hormone insulin. Most commonly, type 2 diabetes is associated with obesity whereas type 1 diabetes is largely a result of immune-mediated destruction of the β-cell. One rare but significant cause of type 2 diabetes is excess blood glucocorticoid levels (Cushing's syndrome). High circulating glucocorticoids potently induce metabolic disorders including peripheral insulin resistance in key metabolic tissues (muscle, liver and fat) as well as directly suppressing β-cell function and can precipitate type 2 diabetes. However, in common forms of metabolic syndrome (visceral obesity, type 2 diabetes, increased cardiovascular disease risk) it appears that amplification of local tissue glucocorticoid action by increased levels of the intracellular enzyme 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), particularly in adipose tissue, is a key driver of the adverse metabolic phenotype rather than altered circulating glucocorticoid levels. 11β-HSD1 is also elevated in pancreatic islets from obese rodents. This thesis aimed to determine the role of 11β-HSD1 in pancreatic islets (β-cells) under normal conditions and its potential pathogenic role in the development of diabetes.

We first determined that 11β-HSD1 acted primarily as a reductase (amplifying glucocorticoid action) in pancreatic islets. We then determined that islet 11β-HSD1 transcription is under the control of the promoters that express in other tissues like liver. Islet 11β-HSD1 is significantly regulated by factors relevant to the diabetic state; high glucose and insulin suppressed whereas fatty acids and TNFα increased 11β-HSD1 activity. To test whether the high islet 11β-HSD1 found in obese rodents was directly diabetogenic, we generated transgenic mice specifically overexpressing β-cell 11β-HSD1 under the mouse insulin promoter (MIP-HSD1 mice) in a mouse strain prone to develop β-cell failure when subjected to diabetic challenge (eg. chronic high fat feeding). Unexpectedly, MIP-HSD1$^{tg/+}$ mice (expressing ~2 fold elevated 11β-HSD1 activity) exhibited markedly improved β-cell insulin secretory

responses, whereas MIP-HSD1$^{tg/tg}$ mice had partially impaired β-cell insulin secretory function *in vivo* and *in vitro*. Moreover, MIP-HSD1$^{tg/+}$ mice completely resisted the mild hyperglycaemia induced by multiple-low doses of the β-cell toxin streptozotocin (40mg/kg i.p. for 5 days) and partially resisted the profound hyperglycaemia induced by a single high dose of streptozotocin (180mg/kg). Notably, MIP-HSD1$^{tg/+}$ mice exhibited lower macrophage infiltration (MAC-2) and higher T-regulatory cell (Foxp3) infiltration after these challenges with evidence of increased insulin-positive cells and maintenance of normal levels of proliferation-competent β-cells. Overall, MIP-HSD1$^{tg/tg}$ exhibited a partial protection from the streptozotocin challenge.

Modestly increased 11β-HSD1 expression in β-cells unexpectedly supports compensatory insulin hypersecretion preventing type 2 diabetes and protects β-cells from inflammatory mediated damage in the setting of type 1 diabetes. Above a protective threshold, elevated β-cell 11β-HSD1 may result in β-cell dysfunction and diabetes. These findings have important implications for the currently advocated therapeutic strategies to inhibit 11β-HSD1 in the context of obesity and diabetes.

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3-MeA	N^3-methyladenine
6-MeG	O^6-methylguanine
7-MeG	N^7-methylguanine
11β-HSD	11 beta-hydroxysteroid dehydrogenase
11DHC	11-Dehydrocorticosterone
ACTH	Adrenocorticotrophic hormone
ADX	Adrenalectomy
ANOVA	Analysis of variance
AP-1	Activator protein-1
BER pathway	Base Excision Repair pathway
bp	Base pairs
BSA	Bovine serum albumin
CBG	Corticosterone binding globulin
C/EBP	CCAAT/enhancer-binding protein
CHOP	CCAAT/enhancer-binding protein homologous protein
CNS	Central nervous system
CRH	Corticotrophin releasing hormone
DAB	Diaminobenzidine
DC	Dendritic cell
DEPC	Diethylpyrocarbonate
DMEM	Dulbeco's modified Eagle's medium
DNA	Deoxyribo-nucleic acid

DTT	Dithiothriotol
EDTA	Ethylene diamine tetraacetic acid
FCS	Foetal calf serum
FFAs	Free fatty acids
FITC	Fluorescein isothiocyanate
Foxp3	X-linked forkhead/winged helix transcription factor
G6P	Glucose-6-phosphate
G6Pase	Glucose-6-phosphatase
G6PT	Glucose-6-phosphate transporter
Gal-3	Galectin-3
GC	Glucocorticoid
GLUT2	Glucose transporter 2
GSIS	Glucose stimulated insulin secretion
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H6PDH	Hexose-6-phosphate dehydrogenase
HDL	High-density lipoprotein
Hlxb9	Homeobox gene 9
HPA-axis	Hypothalamic-pituitary-adrenal -axis
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
HRP	Horse radish peroxidase
IF	Immunofluorenscence

IFN	Interferon
IGF	Insulin-like growth factor
IHC	Immunohistochemistry
IL	Interleukin
IRS-2	Insulin receptor substrate
JNK	c-Jun N-terminal kinase
LC-CoA	Long chain acyl-CoA
LPS	Lipopolysachharide
LXR	Liver X receptor
MafA	v-maf musculoaponeurotic fibrosarcoma oncogene A
MafB	v-maf musculoaponeurotic fibrosarcoma oncogene B
MCP-1	Monocyte chemoattractant protein l
MR	Mineralocorticoid receptor
mRNA	Messenger RNA
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NFκB	Nuclear factor-kappa B
Ngn3	Neurogenin 3
Nkx2-2	Homeoprotein transcription factors NKx2-2
Nkx6-1	Homeoprotein transcription factors NKx6-1
NO	Nitric oxide
NOD mice	Non-diabetes mice
OPT	Optical projection tomography

PARP	Poly adenosine 5’diphosphate-ribose polymerase
Pax4	Paired homeodomain transcription factor 4
Pax6	Paired homeodomain transcription factor 6
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDX1	Pancreatic and duodenal homeobox1
PEPCK	Phosphoenol pyruvate carboxykinase
PPARγ	Peroxisome proliferator-activated receptor-γ
Ptf1a	Pancreas transcription factor 1a
RNA	Ribo-nucleic acid
ROS	Reactive oxygen species
RT	Reverse transcription
Sox9	SRY/HMG box 9
STAT	Signal transducers and activators of transcription
SP	Surfactant protein
STZ	Streptozotocin
TF	Transcription factor
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TUNEL	(TdT) mediated dUTP nick-end labeling
T1D	Type 1 diabetes
T2D	Type 2 diabetes
VLDL	Very-low-density lipoprotein

Chapter 1

Introduction

1 Introduction

1.1 Diabetes

Diabetes is a group of metabolic diseases which are characterized by, and diagnosed with, chronic hyperglycaemia (WHO 1985). The classical symptoms caused by chronic hyperglycaemia are polydipsia (increased thirst), polyphagia (increased hunger) and polyuria (frequent urination).

Diabetes was first recorded in 1552 B.C. when the 3rd Dynasty Egyptian physician Hesy-Ra noted the symptom of polyuria. Diabetes which was derived from Greek "diabainein" in reference to the polyuria was constructed by Aretaeus of Cappadocia around the first centry AD (Dobson 1776). The word mellitus that came from Latin to describe the sweet taste of uria was added by Thomas Willis in 1675. Although, over thousands of years, diabetes was described and studied by many ancient nations, it was considered incurable and predicted a short and painful life (Medvei 1993). Not until 1921 did Banting and his colleagues purify insulin from bovine pancreas and subsequently used it to reverse diabetes in humans the following year (Banting et al. 1922). By 1936, the notable two major types of diabetes were clearly distinguished (Himsworth 1936) according to their different characteristics. Type 1 diabetes (T1D) results from a failure to produce insulin and requires life-long injections of insulin (although recent islet transplant therapy has opened the possibility to finally supersede this (Langer 2010; Ludwig et al. 2010)). Type 2 diabetes (T2D) results from peripheral insulin resistance wherein cells fail to sense and respond to insulin properly. At least initially, T2D can be controlled with medications that improve insulin sensitivity before the need to administer insulin.

Despite the availability of treatment, both type 1 and 2 are chronic conditions that usually cannot be cured. The International Diabetes Federation (IDF) reported that 285 million people worldwide or 6.4% of the population will suffer from diabetes by 2010, which is nearly ten times more than reported in 1985 (Shaw et al. 2009). The

condition is affecting more people of working age than previously predicted and no ethnic group has escaped from its increasing prevalence. It is predicted that if the current rate of growth continues, the total number will exceed 439 million in 2030 (Shaw et al. 2009).

1.1.1 Type 2 diabetes

T2D or NIDDM (Non Insulin Dependent Diabetes Mellitus) is by far the most common cause of glucose intolerance, with 90% prevalence among all diabetic patients (Zimmet et al. 2001). It is primarily characterized by three pathological alterations: peripheral insulin resistance, increased hepatic glucose production and relative insulin deficiency (Efendic et al. 1984; DeFronzo et al. 1992; Kahn 1996).

Although T2D has been studied for a long time, the etiology is still unknown in detail. Inheritable genetic and environmental factors, like stress (Surwit et al. 1992), aging (Jack et al. 2004), high fat diet (Lovejoy et al. 2002), and a less active lifestyle (Hu 2003), are involved in the development of T2D (Hamman 1992). Recently, a large genome wide association studies in the human population identified several candidate genes of T2D, the majority of which are associated with β-cell function (Dupuis et al. 2010). About 55% of T2D cases are associated with obesity (Eberhart 2004). Indeed, obesity is considered a strong causal factor for T2D (Camastra et al. 1999), most likely due to excessive adipose tissue acting as a source of chemical signals (hormones and cytokines) that promote insulin resistance and eventually lead to ‘exhaustion’ of the insulin secretory response.

T2D patients can manage their condition through exercise and diet; though many will require medication including insulin to properly control blood glucose levels. It is estimated that 60% or more of T2D incidence could be prevented or delayed by changing life style (Knowler et al. 2002; Lindstrom et al. 2006; Raina Elley and Kenealy 2008).

1.1.2 Type 1 diabetes

T1D or IDDM (Insulin Dependent Diabetes Mellitus) is an autoimmune disease. It is

characterized by high blood and urine glucose levels which results from the permanent destruction or damage of insulin producing β-cell in the islets of Langerhans of the pancreas

Although T1D has been recognized for a long time, its exact cause is still not fully understood. The immunological origin leading to T1D involves in part genetic predisposition (Maximilian 1990) and environmental factors which interact to create the conditions required for disease onset. Many potential environmental factors have been focused on, such as stress (Williams et al. 1990; Ader et al. 1991; Surwit et al. 1992; Durant et al. 1993), food constituents (Elliott and Martin 1984; Daneman et al. 1987; Elliott et al. 1988; Issa-Chergui et al. 1988), viral mediators (Yoon 1990), and bacteria (Sadelain et al. 1990). However, most people who develop T1D are otherwise healthy.

Unlike T2D, T1D is rapidly lethal and there is a need for either multiple daily insulin injections or continuous subcutaneous insulin fusion. Pancreatic or islet transplants have been used to treat T1D clinically, though this approach is still at the experimental trial stage and requires a lifetime of immunosuppression (Langer 2010; Ludwig et al. 2010; Robertson 2010; Kort et al. 2011). A drive to create artificial glucose responsive pancreatic islet tissues is currently at the forefront of T1D research (Opara et al. 2010).

1.2 The pancreas

The pancreas, in particular the β-cell principally maintain blood glucose levels by secreting insulin, a protein which was named by Edward Albert Sharpey-Schafer of Edinburgh in 1910, it was be purified and shown to reverse high glucose effects in dogs by Frederick Grant Banting and Charles Herbert Best at the University of Toronto in 1921. For this Banting and his colleagues got the Nobel Prize in 1923.

1.2.1 The structure and function of the pancreas

The pancreas is a gland organ located across the back of the abdomen, behind the

stomach; the widest right-most side of the pancreas, called the head of pancreas, is connected to the duodenum by the pancreatic duct. The tapered remaining part extending leftward is called the body; the section near the spleen is called the tail of pancreas (Figure 1-1).

The pancreas is both an exocrine and endocrine gland. The exocrine pancreas which corresponds to 98% of the mature organ weight secretes the pancreatic juices consisting of two major components into the duodenum via the pancreatic duct to help digest carbohydrates, protein, and fat. Pancreatic enzymes are secreted by the acinar cells and pass into the small intestine to breakdown dietary protein, fat and carbohydrates in chyme; the other is an aqueous alkaline solution rich in sodium bicarbonate ($NaHCO_3$) which is secreted by duct cells to neutralize excess acidity in the intestines. The endocrine function is performed by islets of Langerhans, a cell cluster that consists of at least five different endocrine cell types. Hormones released from the endocrine cells enter the blood and lead to disposal of the absorbed nutrients (e.g. insulin from endocrine β-cells), or, alternatively, they mobilize nutrients in times of fasting (e.g. glucagon from endocrine α-cells).

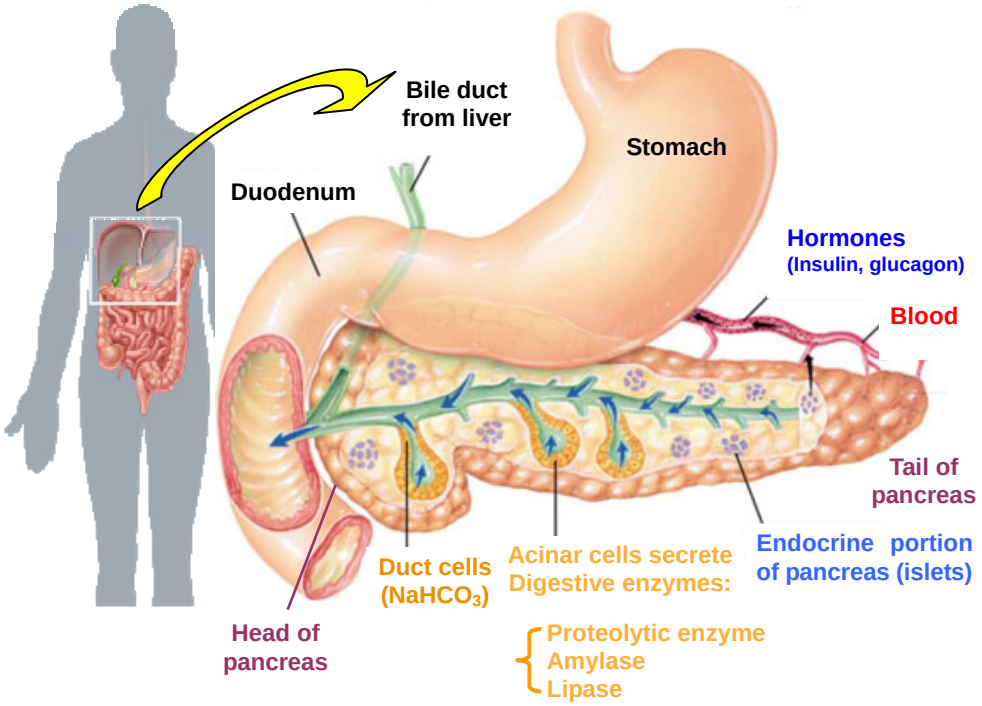


Figure 1-1. The structure of the pancreas. The pancreas is an elongated organ that lies across the back of the abdomen behind and below the stomach. The widest right side of pancreas, called the head, is connected to the duodenum by the pancreatic duct. The end near the spleen is called the tail of pancreas. The exocrine part consists of grape-like clusters of secretory cells that form digestive enzymes known as acinar cells, which connect to the pancreatic duct. A small proportion of the gland consists of isolated islands of endocrine tissue known as islets of Langerhans which are dispersed throughout the pancreas and arranged as cords among the capillary channels. (Picture modified from University of Colorado at Boulder and 2009 WebMD, LLC.)

1.2.2 Pancreatic islets of Langerhans

Islets of Langerhans are cell clusters (often hundreds of cells) that are spread throughout the pancreas and perform the endocrine function (Henderson 1969). The islets consist of five major cell types which are characterized by different hormone secretion profiles (Figure 1-2): β-cells secrete insulin (to decrease blood glucose); α-cells secrete glucagon (to increase hepatic glucose production and blood glucose); δ-cells secrete somatostatin (to regulate α and/or β-cell hormonal secretion); PP cells secrete pancreatic peptide (to inhibit gall bladder contraction, increase gastrointestinal motility and self regulate pancreatic exocrine and endocrine secretion), and Epsilon cells secrete ghrelin (to increase hunger before a meal, stimulate growth hormone secretion and inhibit fat utilization in adipose tissue).

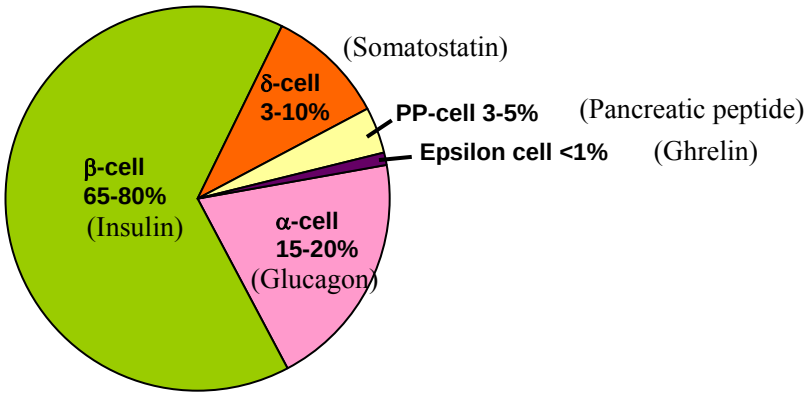


Figure 1-2. Cell components of the islet of Langerhans. Islets of Langerhans constituted of at least five main cell types secreting different hormones.

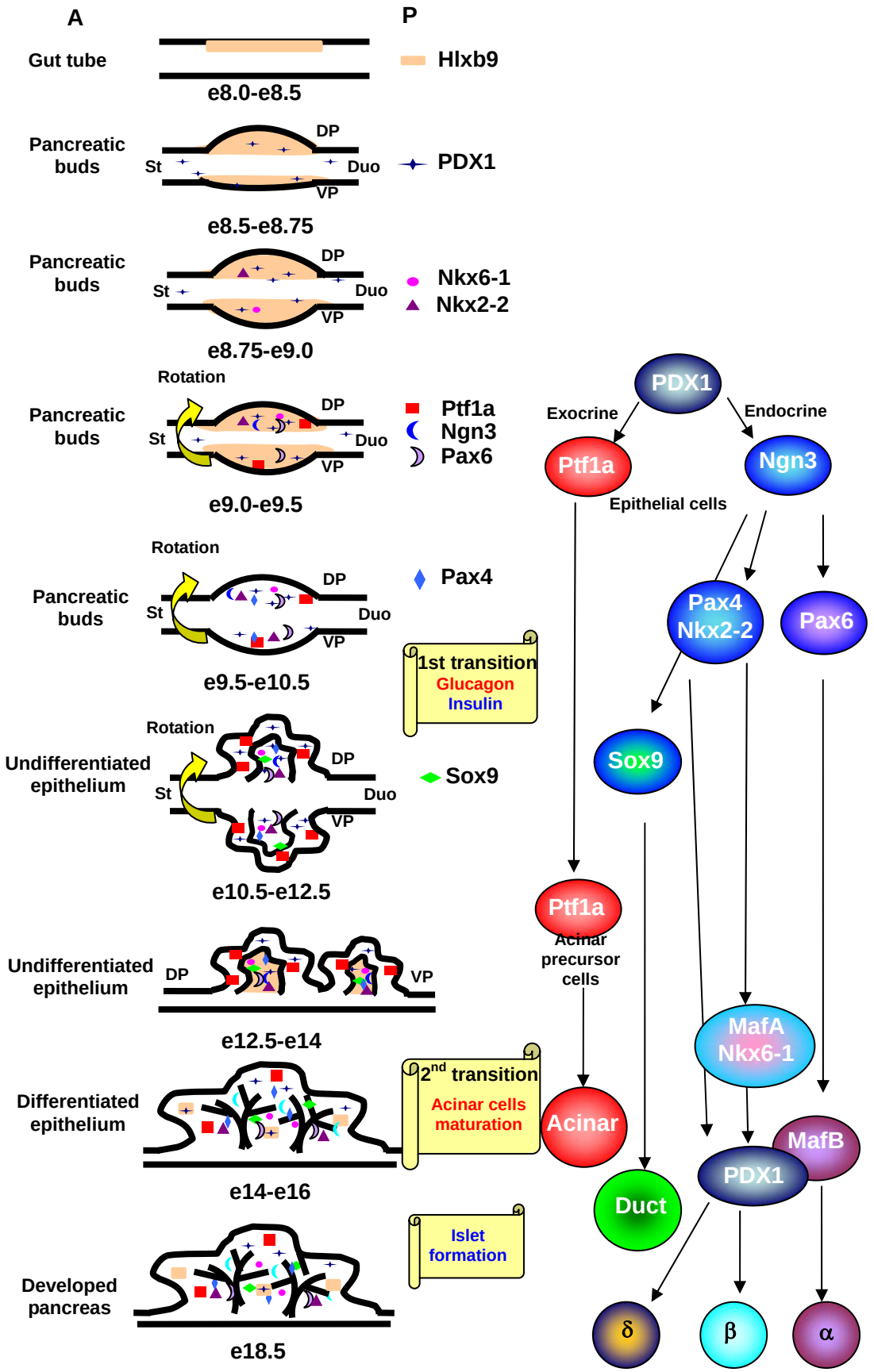
1.2.3 Anatomical and morphological origins of the pancreas and progenitor cell development

Studies of pancreatic developmental biology have shed new light on the cause of T2D. As the work involved in this thesis relates to the mouse, this section focuses on mouse pancreatic development. The origin of the pancreas is from the embryonic endoderm foregut which locates anterior to the duodenum and posterior to the developing liver. Pancreatic development starts from embryonic day e8.5-9.0 with the formation of dorsal and ventral buds. The dorsal bud lies in close proximity to the notochord and lateral to the dorsal aorta. The ventral bud comes from the hepatic diverticulum and becomes the head. In mice, at e9.5, the dorsal and ventral buds begin a differential rotation. The duodenum rotates to the right along with the ventral bud. By e10.5, the partially differentiated epithelium of the two buds undergoes branching morphogenesis into a ductal tree that by e12.5 results in the formation of two primordial pancreas organs consisting predominantly of an undifferentiated ductal epithelium. Between e13 to e14, the ventral bud fuses with the much larger dorsal bud to form a single definitive pancreas (Carlson 2004). (Figure 1-3)

Mouse pancreatic endocrine progenitor cells are present from the very beginning of development (e9.5) and proceed through a first and secondary transition (Rutter et al. 1968). At e9.5, before epithelial branching, glucagon and insulin can be detected within the developing pancreatic bud which is considered as the first developmental transition (Teitelman et al. 1993). The secondary transition happens at approximately e13-16 when a dramatic increase in the number of endocrine cells budding from the ductal epithelium begins, and they organise into islet-like clusters. The islets are not fully formed until e18.5 when the differentiated endocrine cells begin to separate from the ductal epithelium and migrate to the acinar-rich area then spontaneously organise into islets. During e14.5-e15.5, the exocrine pancreas differentiates from the ductal epithelium; on e15.5, acinar cells are clearly discernible from ducts. (Figure 1-3)

Figure 1-3. Schematic represention of morphogenetic events and expression of transcription factors during embryonic pancreas development. Hlxb9 is expressed in dorsal endoderm before initiation of DP organogenesis; PDX-1 appears between e8.5 and e8.75 at the foregut domain, VP begins to form; Nkx2-2 coexpresses with PDX-1 at e8.75, Nkx6-1 expresses at VP; by e9.0 some cells turn on Ngn3 expression and go through the endocrine pathway, some cells expressing Ptf1a and co-expressing PDX-1 enter the exocrine pathway; at e9.5, VP starts to rotate to the right, several transcription factors co-express at DP and Pax4 appears. At this stage, glucagon and insulin can be detected (first transition); from e10.5 to e12.5, the uniform epithelial structure of the entire pancreatic epithelium becomes highly branched, and by e12.5, the undifferentiated ductal epithelium forms. Nkx2-2, Nkx6-1, Hlxb9-positive cells segregate to the central epithelium, whereas Ptf1a-positive cells are specifically found in the periphery and migrate to regulate acinar cell maturation; from e12.5 to e14, the VP fuses with DP to form a single pancreas organ; the buds begin to differentiate into endocrine and exocrine cellular lineages by e14 and proliferate and expand extensively, acinar cells complete maturation; from e13.5 to e14.5 (second transition) the endocrine cells organize into isolated clusters that condense into the islets of Langerhans by e18.5.

Abbreviations: A, anterior; P, posterior; st, stomach; duo, duodenum; DP, dorsal pancreas; VP, ventral pancreas. Adapted from (Jorgensen et al. 2007).


A
P
Gut tube
e8.0-e8.5
Hlxb9
Pancreatic buds
DP
St
Duo
VP
e8.5-e8.75
PDX1
Pancreatic buds
e8.75-e9.0
Nkx6-1
Nkx2-2
Rotation
Pancreatic buds
e9.0-e9.5
Ptf1a
Ngn3
Pax6
Rotation
Pancreatic buds
e9.5-e10.5
Pax4
1st transition
Glucagon
Insulin
Rotation
Undifferentiated epithelium
e10.5-e12.5
Sox9
Undifferentiated epithelium
e12.5-e14
Differentiated epithelium
e14-e16
2nd transition
Acinar cells maturation
Developed pancreas
e18.5
Islet formation
PDX1
Exocrine
Endocrine
Ptf1a
Ngn3
Epithelial cells
Pax4
Nkx2-2
Pax6
Sox9
Ptf1a
Acinar precursor cells
MafA
Nkx6-1
Acinar
MafB
PDX1
Duct
δ
β
α

1.2.4 Essential transcription factors for pancreatic development in the embryo

Certain key transcription factors are involved in the regulation of the genes expressed in both developing and adult pancreas. Disruption of these genes results in impaired pancreatic development and consequent diabetes. Transcription factors often serve dual functions in determining early cellular development and later in maintaining the phenotype of terminally differentiated cells. At an early stage, PDX1, Ptf1a and Hlxb9 are required for pancreatic bud development. Later, specific transcription factors are activated or inhibited, directing the pancreatic cell differentiation and proliferation towards one lineage or another, corresponding to the exocrine and endocrine functions of the pancreas. For example, the notch receptor system regulates progenitor cell differentiation to exocrine pancreas via expression of Ptf1a, whereas differentiation of the endocrine pancreas in the absence of notch receptor signaling occurs under the influence of Ngn3. The known essential transcription factors are listed in Table 1-1 and Figure 1-3.

Table 1-1. Essential transcription factors in pancreatic development

Transcription factors	Expression in embryologic stage	Characteristics	Adult expression region	References
Hlxb9/Hb9 (Homeobox gene 9)	Starts at e8.0, disappears at e10, reappears at e12.5	Essential factor for dorsal pancreatic specification	β-cell	(Harrison et al. 1999; Li et al. 1999)
MafA (v-maf musculoaponeurotic fibrosarcoma oncogene A)	Expresses in insulin positive cells after 2nd transition.	An activator of insulin gene expression; Marker for mature β-cell, specific for developing and maturated β-cell.	β-cell	(Olbrot et al. 2002; Matsuoka et al. 2004)
MafB (v-maf musculoaponeurotic fibrosarcoma oncogene B)	Expresses in both insulin and glucagon cells before 2nd transition, and then restricts in glucagon cells at e15.	Marker for mature α-cell.	α-cell	(Artner et al. 2006; Artner et al. 2007)
Ngn3 (Neurogenin 3)	Starts at e9–e9.5, peaks on e15.5, diminishes at birth	Essential for all endocrine cell development; Marker for islet cell precursor cells.	Little or undetectable	(Gradwohl et al. 2000; Jensen et al. 2000; Johansson et al. 2007)
Nkx2-2 (Homeoprotein transcription factors NKx2-2)	Starts at e8.75 at DP, e9.5 at VP, coexpresses with Ngn3 at e10.5	Regulation of the differentiation of pancreatic endocrine cells; Essential for β-cell differentiation.	α-cell, β-cell, PP cell	(Sussel et al. 1998; Sander et al. 2000; Wang et al. 2004)
Nkx6-1 (Homeoprotein transcription factors NKx6-1)	Starts at e8.75 at VP, e9.0 express at DP, not VP, e10.5 reappear at VP, highly expresses at e13.5 to e14.5	Marker for mature β-cell; Regulation of the differentiation of pancreatic endocrine cell;	β-cell	(Jensen et al. 1996; Sander et al. 2000)
Pax4 (Paired homeodomain transcription factor 4)	Starts at e9.5, peaks at 2nd transition, coexpresses with Ngn3	Promotes the developing islet cells differentiation of β-and δ-cell.	β-cell	(Sosa-Pineda et al. 1997; Wang et al. 2004)
Pax6 (Paired homeodomain transcription factor 6)	Starts at e9.0, peaks at e9.25, expresses in cluster at e11.5, which predominant at e12.5.	Early marker for endocrine cells; Regulates the terminal steps in cellular differentiation of the endocrine pancreas;	α-cell (predominant), β-cell, δ-cell, PP cell	(Sander et al. 1997; St-Onge et al. 1997; Andersen et al. 1999)

		Regulates the promoters of the glucagon, insulin, and somatostatin genes,		
PDX-1/ IPF1/IDX-1 (Pancreatic duodenal homeobox1)	Starts at e8.5, after e12.5 the expression decreases, then highly re-expresses at e13.5, more prominent at e14.5.	Critical transcriptional activator of insulin and somatostatin in adult islets; Necessary in the fate of endocrine cell types; Important in determining β-cell maturation and function.	Insulin (β-cell) Somatostain (δ-cell)	(Ohlsson et al. 1991; Ohlsson et al. 1993; Offield et al. 1996; Li et al. 1999; Gannon et al. 2008; Liew et al. 2008)
Ptf1a/p48 (Pancreas transcription factor 1a)	Starts from e9.0-e9.5, migrates to acinar cells at e12.5.	Important transcription factor of acinar cells; Regulates exocrine tissue gene transcription.	Acinar cells	(Krapp et al. 1996; Rose et al. 2001)
Sox9 (SRY/HMG box 9)	Starts at e10.5, co-expresses with Ngn3 at e15.5.	Essential factor for pancreatic growth and endocrine cell differentiation.	Ductal cells	(Lynn et al. 2007; Seymour et al. 2007)

1.3 The pancreatic β-cell

The β-cells are most remarkable for their exquisitely regulated secretion of insulin. Both type 1 and type 2 diabetes are linked with β-cell dysfunction or loss.

1.3.1 Pancreatic β-cell regeneration

The specialized features of the β-cell are determined by the expression of a subset of genes controlled by a number of transcription factors, some of which are β-cell-specific and others are ubiquitous in adult mouse tissue as described before in Table 1-1.

Shortly after birth the endocrine pancreas undergoes remodelling through a process that involves substantial apoptosis and β-cell replication (Bonner-Weir 2000). The first wave of new islet formation is in the first few days after birth, the second wave happens at weaning (Scaglia et al. 1995). In adult mouse, under normal conditions, the β-cell mass remains linearly related to body weight and replication is the major mechanism for the growth in β-cell number (Bonner-Weir 2000). There are small changes in β-cell number from 30 to 40 days after birth in the rat (Finegood et al. 1995). The regulation of β-cell growth and proliferation occurs mainly through insulin/IGF signalling acting through IRS-2 (Augstein et al. 1998; Kubota et al. 2000). Although the postnatal β-cell replication rate is low, like most other cell types β-cells have a finite lifespan. Mathematical modelling of the lifespan of rat β-cells has estimated they have a slow turnover rate with a half-life estimated at 30-60 days in young rats (Finegood et al. 1995). Physiological apoptosis is a mechanism for β-cell turnover and this can be observed *in vitro* (Rabinovitch 1994). Further, β-cell apoptosis occurs during the involution of the β-cell mass postpartum *in vivo* (Scaglia et al. 1995) as well as in pancreas remodelling in the neonatal rat (Scaglia et al. 1997).

1.3.2 Pancreatic β-cell function

β-cells store a large amount of insulin in mature granules and release a small proportion of these granules immediately following glucose stimulation. To renew these stores, insulin biosynthesis is immediately stimulated and occurs mainly at the translational level (Itoh and Okamoto 1980; Welsh et al. 1986). Under physiological conditions, the quantity of insulin released by β-cells is directly related to the prevailing glucose concentration, which may depend on the nature, quantity and route of administration or exposure to nutrients.

1.3.2.1 Insulin transcription, synthesis and release

Insulin is an important hormone in regulating metabolic homeostasis. Preproinsulin which is the first product of insulin mRNA translation is encoded by a single gene in most species including human (Harper et al. 1981). In rodents there are two non-allelic insulin genes present. In rats, insulin gene I and II are colocalised on the same chromosome 1 (Soares et al. 1985). In mouse, the insulin gene I is located on chromosome 19 (Davies et al. 1994), and the insulin gene II is localised on chromosome 7 (Duvillie et al. 1998). Physiological insulin expression is restricted to pancreatic β-cells, although the insulin gene I is also found moderate expression in rat brain predominantly in hippocampus (Gerozissis 2003). Insulin transcription is controlled by the insulin promoter that is a highly conserved ~340 bp region located upstream from the transcription start site (Poitout et al. 2006). The insulin promoter binds β-cell specific as well as ubiquitous transcription factors (German et al. 1995; Poitout et al. 2006). The most critical transcription activation elements of the insulin promoters are the E, A and C sites ("boxes") (German et al. 1995) as well as additional sequences that may have more subtle regulatory effects.

The E boxes bind proteins of the basic helix–loop–helix (bHLH) class of transcription factors. In β-cells, the ubiquitous bHLH protein E47 forms a heterodimer with NeuroD/BETA2 (β-cell E box transactivator 2, β2) which is expressed in neuroendocrine cells (Naya et al. 1995). There are up to five A box sequences within the insulin promoter. Their consensus TAAT sequence is a known

binding site for PDX-1 (Boam and Docherty 1989; Leonard et al. 1993; Ohlsson et al. 1993; Miller et al. 1994; Marshak et al. 1996). PDX-1 interacts synergistically with E47/β2 (Peers et al. 1994; Peshavaria et al. 1997; Glick et al. 2000; Ohneda et al. 2000) and this activates the insulin promoter. Histones and DNA-binding proteins of the high mobility group (HMG) HMG 1(Y) (Ohneda et al. 2000) can increase the binding of PDX-1 to the A and E sites. Another important transcription factor is MafA which belongs to the Maf (musculoaponeurotic fibrosarcoma oncogene) family and binds to the C1 site of the insulin promoter. MafA is a glucose-regulated and pancreatic β-cell-specific transcriptional activator of the insulin gene (Kataoka et al. 2002; Kataoka et al. 2004; Matsuoka et al. 2004). Members of the hepatic nuclear factors (HNF) and PAX families (Paired homeodomain transcription factor) (Melloul et al. 2002) also contribute to insulin transcription. Insulin gene transcription is regulated through the activation of these factors and in combination, these factors can exert strong stimulatory effects (Glick et al. 2000; Ohneda et al. 2000; Aramata et al. 2005; Zhao et al. 2005).

After translation of the preproinsulin mRNA, the "pre" portion of preproinsulin is removed by a proteinase, and proinsulin is transported in transport vesicles along the microtubule network system to the Golgi apparatus and packaged into clathrin-coated immature granules. Proinsulin is further converted to insulin and C-peptide by trypsin- and carboxypeptidase-like enzyme (Orci et al. 1987). The clathrin-coated granules then become mature granules where the insulin crystals are assembled in close association with Zinc and Calcium, and finally stored as a hexamer which is inactive (Coffman and Dunn 1988; Kadima et al. 1992). Insulin is released by exocytosis after glucose stimulation. The granules carrying insulin hexamers are released into the intercellular space where they dissolve to expose insulin monomers which are the active form of insulin (Orci et al. 1984).

1.3.2.2 The role of glucose in insulin secretion

Glucose is the main physiologic regulator of insulin transcription, translation and secretion. Basal insulin gene transcription occurs at low plasma glucose concentrations (< 5.6mM). The transcription of insulin increases at higher glucose

concentrations, and is sensed through increased glucose metabolism by the high K_m glucose transporter 2 (GLUT2) and glucokinase (GK) activity within the β-cell (Newgard and McGarry 1995). Glucose-induced insulin transcription is modulated by many factors which influence insulin transcription as mentioned above, and the insulin promoter boxes.

β-cells release insulin in response to glucose metabolism (Maechler et al. 2006). After entering the β-cell through the GLUT2 transporter, glucose is phosphorylated to glucose-6-phosphate (G6P) by glucokinase and metabolised via glycolysis to generate pyruvate which is then utilised by the mitochondria (Newgard and McGarry 1995; Prentki 1996; Nolan et al. 2006). In the glucose oxidation pathway, approximately 50% of pyruvate is metabolised by pyruvate dehydrogenase producing acetyl-CoA which enters the tricarboxylic acid cycle (TCA) to generate ATP. This causes an increased intracellular ATP/ADP ratio (Newgard and McGarry 1995; Prentki 1996; Schuit et al. 1997) which results in closure of the plasma membrane K_{ATP}-channels that induces membrane depolarization, and triggers increased calcium concentrations in the cytoplasm through opening of voltage-gated calcium channels (Ashcroft and Rorsman 1990). The increased intracellular calcium level induces the insulin secretory granules to fuse with the membrane and release insulin by exocytosis (MacDonald and Wheeler 2003). This pathway, often termed the K_{ATP} channel-dependent pathway is considered to be the major triggering event for glucose stimulated insulin secretion (GSIS). Pyruvate can also be metabolised by pyruvate carboxylase to oxaloacetate, which ensures the provision of a carbon skeleton to the TCA cycle in the mitochondria, a process known as anaplerosis (Schuit et al. 1997; Brennan et al. 2002; Liu et al. 2002). (Figure 1-4)

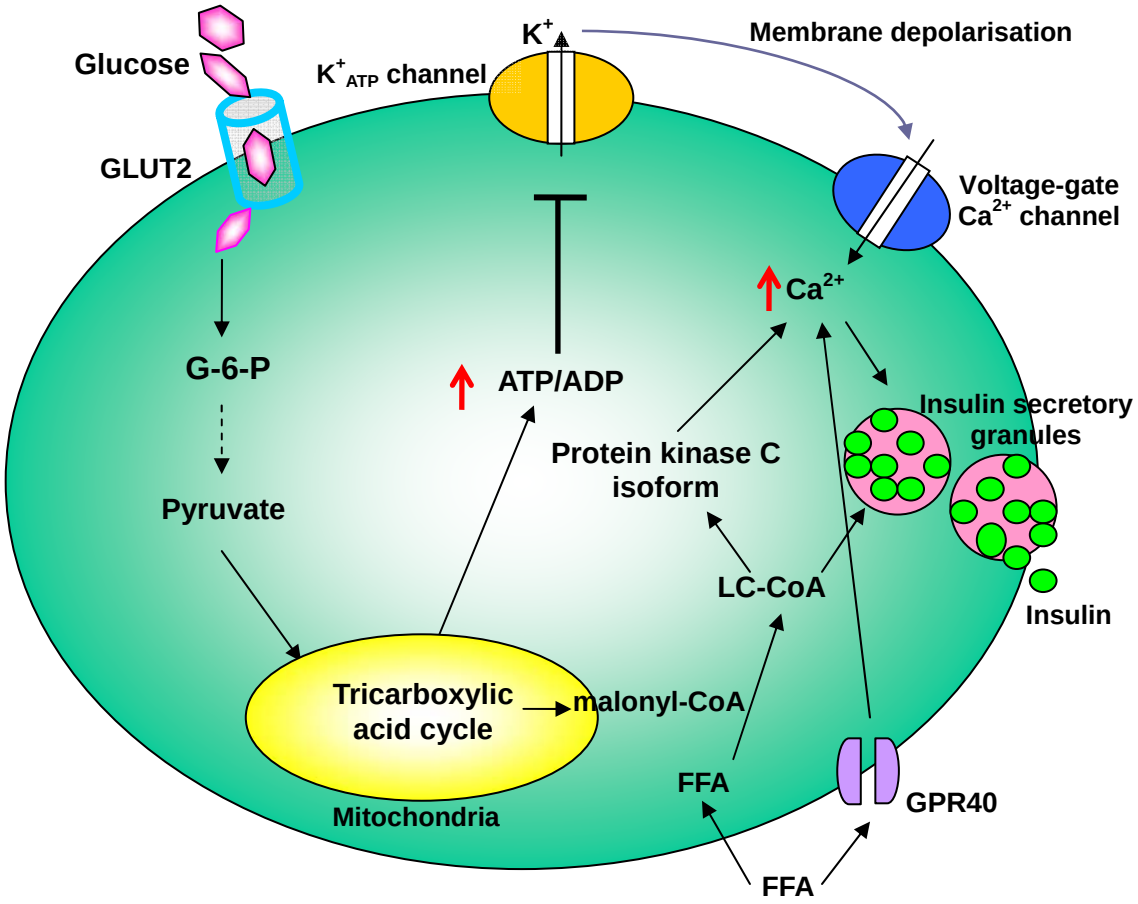


Figure 1-4. Glucose regulates insulin secretion. Glucose enters in the β-cell through the GLUT2 transporter. Glucose is metabolized through glycolysis and the tricarboxylic acid cycle to generate ATP, resulting in an increased intracellular ATP/ ADP ratio, which cause closure of K_{ATP}-channels. This depolarizes the plasma membrane and increases calcium concentrations in the cytoplasm through voltage-gated calcium channels. The increased calcium causes insulin secretory granules to be released by exocytosis**.** Fatty acids influence insulin release by both directly activating GPR40 and through formation of malonyl-CoA to generate long chain acyl-CoAs (LC-CoA) which stimulate insulin granule exocytosis, either directly or through PKC-dependent mechanisms.

1.3.2.3 The role of fatty acids in insulin secretion

Free fatty acids (FFAs) are important to the pancreatic β-cell for its normal function, its capacity to compensate for insulin resistance, and its failure in type 2 diabetes (Prentki et al. 2002; Nolan et al. 2006). FFAs alone do not stimulate insulin secretion, however, they potentiate insulin release in response to glucose (Stein et al. 1996; Dobbins et al. 1998). This might involve two different mechanisms. The first relates to the binding of FFAs to the G protein-coupled receptor GPR40 which is a transmembrane receptor highly expressing on the islet β-cell membrane (Itoh et al. 2003). Activation of GPR40 by FFAs outside the β-cell causes the activation of intracellular signalling and a subsequent increase in intracellular calcium levels, and secretory granule exocytosis (Itoh et al. 2003; Shapiro et al. 2005). GPR40 contributes approximately half of the effect of FFAs to augment GSIS. The other mechanism involves malonyl-CoA formation during anaplerosis. Malonyl-CoA forms as a consequence of citrate exit from mitochondria which blocks carnitine palmitoyltransferase 1 (CPT-1), inhibiting the removal of long chain acyl-CoAs (LC-CoA) via their mitochondrial β-oxidation (Prentki and Corkey 1996; Roduit et al. 2004; Nolan et al. 2006). This permits the generation of LC-CoA that then increases insulin secretion both by directly stimulating secretory granule exocytosis and by protein kinase C (PKC) activation (Prentki et al. 2002). (Figure 1-4)

1.3.3 The β-cell and diabetes

Plasma glucose levels are tightly controlled by the interplay of tissue insulin sensitivity, insulin independent glucose metabolism and pancreatic β-cell insulin release. There is a feedback loop between the insulin-sensitive tissues and the β-cell, with β-cells increasing insulin supply in response to demand by the liver, muscles and adipose tissue (Kahn et al. 1993). Failure of this feedback loop results in a deviation from normal glucose tolerance and underlies the development of diabetes. T2D subjects lack the first phase of insulin secretion, which constitutes the initial glucose sensing response of the pancreatic β-cell to altered circulating glucose concentrations.

1.3.3.1 β-cell failure

Unlike other cell types, the β-cell can not protect itself by blocking uptake of excess nutrients (particularly glucose and FFAs). Rather, the β-cells consistently enhance insulin secretion, responding to chronically increased glucose and FFA loading, so that excess glucose and FFA can be stored as glycogen in liver and triacylglycerols (TG) in adipose tissue, respectively. This adaptation allows maintainenance of normal glycaemia even in individuals that are insulin-resistant in several metabolic tissues (liver, adipose tissue, muscle). In this β-cell hyperinsulinaemic compensation stage the increased metabolism within the β-cell leads to high levels of oxidative damage and the ER stress response.

The ER is a critical intracellular organelle with a vast network of membranes which plays an important role in the biosynthesis of secretory proteins, assembling the secretory and membrane proteins into secondary and tertiary structures, and is an important reservoir for Ca^{2+} (Nielsen and Petersen 1972; Chevet et al. 2001). Any misfolded or unfolded proteins are removed and degraded through the ER (Schroder and Kaufman 2005; Marciniak and Ron 2006). When there is an accumulation of unfolded proteins, energy and nutrient fluctuation, or a disruption of Ca^{2+} homeostasis, increased perturbations in the ER occur and activate the ER stress

response, known as the unfolded protein response (UPR) (Schroder and Kaufman 2005; Marciniak and Ron 2006). When this response fails to preserve and restore ER function, cell apoptosis occurs (Marciniak and Ron 2006; Eizirik et al. 2008).

Under physiological conditions, the ER is highly developed in β-cells to maintain their continuous insulin secretory potential. Due to their glucose responsive, highly oxidative nature, the β-cell is susceptible to the accumualtion of mis/unfolded and premature proteins which are the consequences of reactive oxygen species (ROS) (Kaneto et al. 2005) and excessive nitrous oxide (NO) (Oyadomari et al. 2001), which results in ER stress (Harding et al. 2001; Weir et al. 2001). Indeed ER stress in β-cell is induced by FFA in obesity and T2D (Ozcan et al. 2004). The increased ER stress activates the JNK and CHOP pathway (Oyadomari et al. 2001; Kaneto et al. 2005), both of which are involved in β-cell apoptosis. Initially, increased ER stress responses in the β-cell may preserve β-cell function to compensate for the apoptotic loss of β-cells. However, finally, exaggerated ER stress responses lead to β-cell dysfunction (Weir et al. 2001; Poitout and Robertson 2002).

β-cell failure occurs when islets are unable to sustain β-cell compensation for insulin resistance and may result from inadequate expansion of β-cell mass or failure of the existing β-cells to respond to glucose. Glucotoxicity (Unger and Grundy 1985), lipotoxicity (Unger 1995), and glucolipotoxicity (Poitout and Robertson 2002; El-Assaad et al. 2003) have all been proposed to increase the risk of β-cell damage from mitochondrial or ER stress. When β-cell failure is present, impaired glucose tolerance, elevating fasting glucose and type 2 diabetes result. Furthermore, the natural history of type 2 diabetes entails a progressive deterioration in β-cell function (Kahn 2001) associated with β-cell loss by apoptosis (Butler et al. 2003; Yoon et al. 2003) and characteristic functional impairment of the first-phase secretory response. (Figure 1-5)

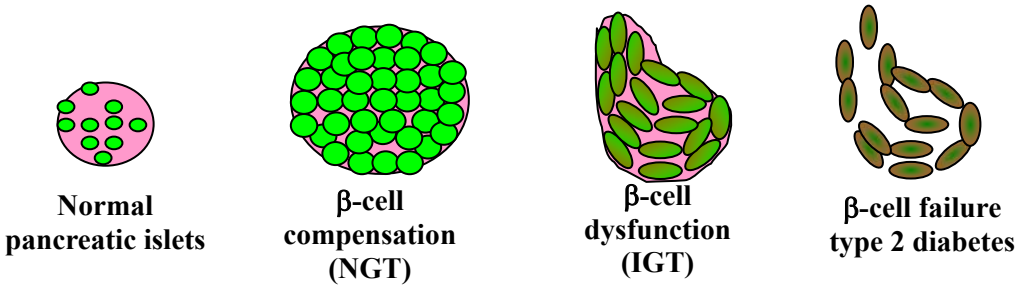


Figure 1-5. β-cell failure and the natural history of Type 2 Diabetes. Type 2 diabetes is associated with both insulin resistance (and/or hyperinsulinemia) and β-cell dysfunction. A progressive decline in β-cell function leads to loss of islet β-cell compensation for the insulin resistance. As a consequence, progress from normal glucose tolerance (NGT) to impaired glucose tolerance (IGT) and finally to established type 2 diabetes occurs. Even after diagnosis of type 2 diabetes, β-cell function continues worsening and eventually insulin can not maintain glucose homeostasis. Green circles represent normal β-cells that increase in number (NGT), then becomes dysfunctional (hypertrophic β-cell: dark green oval shapes, IGT) and finally undergo failure (failing β-cells: brown oval shapes with green central spots). Picture adapted from (Prentki and Nolan 2006).

1.3.3.2 β-cell compensation

In the diabetic condition, hyperglycaemia initially increases both β-cell function (Chen et al. 1994) and islet mass (Bonner-Weir and Smith 1994; Alonso et al. 2007) as a protective action. The adult islet mass expansion increases islet volume but not numbers (Bock et al. 2003), with hypertrophy of existing β-cells (Jonas et al. 1999). However, this view was challenged by the finding that β-cell proliferation was a major source of new β-cells under both normal physiological conditions and after 70% or 50% pancreatectomy (Dor et al. 2004; Teta et al. 2007). PDX1 acts as a critical regulator of β-cell replication that occurs during the compensatory response to insulin resistance (Kulkarni et al. 2004). Generation of β-cells from undifferentiated or partially differentiated precursors may also be involved in the maintenance of β-cell mass, a process called neogenesis (Xu et al. 2006). Although, very little is known about these putative stem cells, several cell types are proposed as possible pancreatic progenitors, such as the acinar cells (Zhou et al. 2008; Sangiorgi

and Capecchi 2009); ductal epithelial cells (Bonner-Weir 2000; Xu et al. 2008); centroacinar cells which lie at the junction between acinar cells and the adjacent terminal ductal epithelium (Rovira et al. ; Hayashi et al. 2003; Nagasao et al. 2003); mesenchymal-like nestin-expressing cells (Zulewski et al. 2001) and intra-islet δ cells (Fernandes et al. 1997).

1.4 Glucocorticoids

Glucocorticoids (GCs) are a group of corticosteroid hormones which are synthesised primarily in the adrenal gland. The primary glucocorticoid in man and most mammals is cortisol, whereas in rodents and lower vertebrates it is corticosterone.

GCs were first discovered in the 1940s and in the years that followed, synthetic GCs soon became one of the most effective medicines to treat inflammation (Hench et al. 1949). GCs have pleiotropic effects on physiological functions in the body including those involved in energy balance and metabolism, immune function, cardiovascular function, circadian rhythmicity, growth and development, cognitive processes and the modulation of various types of behaviour. For example, GCs regulate energy storage by participating in glycogenic and glycolytic pathways (Villar-Palasi and Larner 1970; Nyirenda and Seckl 1998). GCs are also a powerful immunosuppressant in high doses, which led to the development of therapeutic applications for the treatment of asthma (reviewed in Stellato 2007) and rheumatoid arthritis (reviewed in Gaffo et al. 2006). In foetal development, GCs are necessary for survival at birth: mice with disruption of the glucocorticoid receptor (GR) gene die at birth because of a lack of normal lung maturation (Cole et al. 1995; Nemati et al. 2008).

1.4.1 Glucocorticoid synthesis and secretion

Endogenous GCs are produced mainly in the adrenal gland from the common precursor cholesterol through a series of enzymatic reactions which are located in either the smooth endoplasmic reticulum or mitochondria. The basic structure of cholesterol is maintained throughout the steroid biosynthetic pathways which contain a cyclopentane ring and 3-cyclohexane rings. Specifically, GCs are synthesized in the adrenal cortex and are secreted into the circulation (de Kloet 2003). (Figure 1-6)

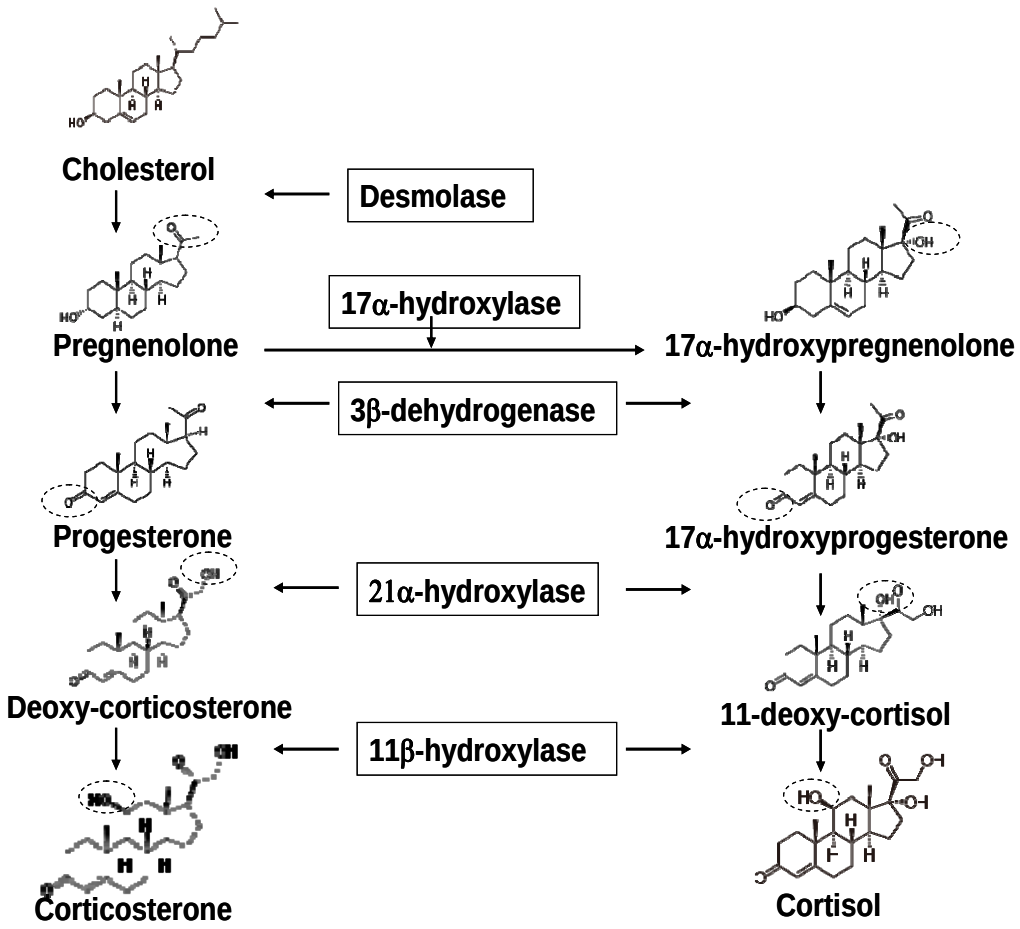


Figure 1-6. Corticosteroid bio-synthesis. In the adrenal gland, humans synthesize cortisol from cholesterol, whereas, rodents synthesize corticosterone as they lack 17α-hydroxylase. Diagram adapted from O'Riordan JLH (1982).

Circulating GC levels are regulated by a negative feedback loop: the hypothalamus-pituitary-adrenal gland (HPA) axis (Aguilera 1998). The HPA axis is activated either by a circadian rhythm or stress stimulus. Under non-stress conditions, circulating levels of GCs are released both in an ultradian and diurnal manner. The suprachiasmatic nucleus, which is considered as an endogenous biological 'clock' sets the circadian rhythm by recurring daylight and darkness (Takahashi and Zatz 1982). In mammals, the peak secretion of GCs happens just before the active period, whereas the nadir occurs during the rest (quiescence/sleep) period.

Upon stimulus (e.g. stress, such as pain, trauma or emotional stress), neural inputs which perceive these signals converge at the main HPA axis control centre, the hypothalamus, to initiate adrenocorticotropin hormone (ACTH) secretion. The parvocellular cells of the paraventricular nucleus in the hypothalamus rapidly release corticotropin-releasing hormone (CRH) and arginine vasopressin (AVP) into the portal circulation to target the anterior pituitary where CRH and AVP then act synergistically to stimulate the secretion of stored ACTH into the bloodstream (Figure 1-7). Circulating ACTH binds to its receptor located at the cell surface of the adrenal cortex to stimulate the synthesis and secretion of GCs (Axelrod and Reisine 1984). Meanwhile, GCs exert a negative feedback on the HPA axis by suppressing the production of CRH and ACTH (Ma et al. 1997), to prevent excess synthesis of GCs and restore the normal basal levels (Dallman et al. 2004). Only five percent of active GCs circulate free in the plasma, most are bound to plasma proteins. Approximately 90% of cortisol and 80% of corticosterone is bound to corticosteroid binding globulin (CBG) (Heyns and Coolens 1988), much of the remaining GCs are found associated with albumin (Dunn et al. 1981).

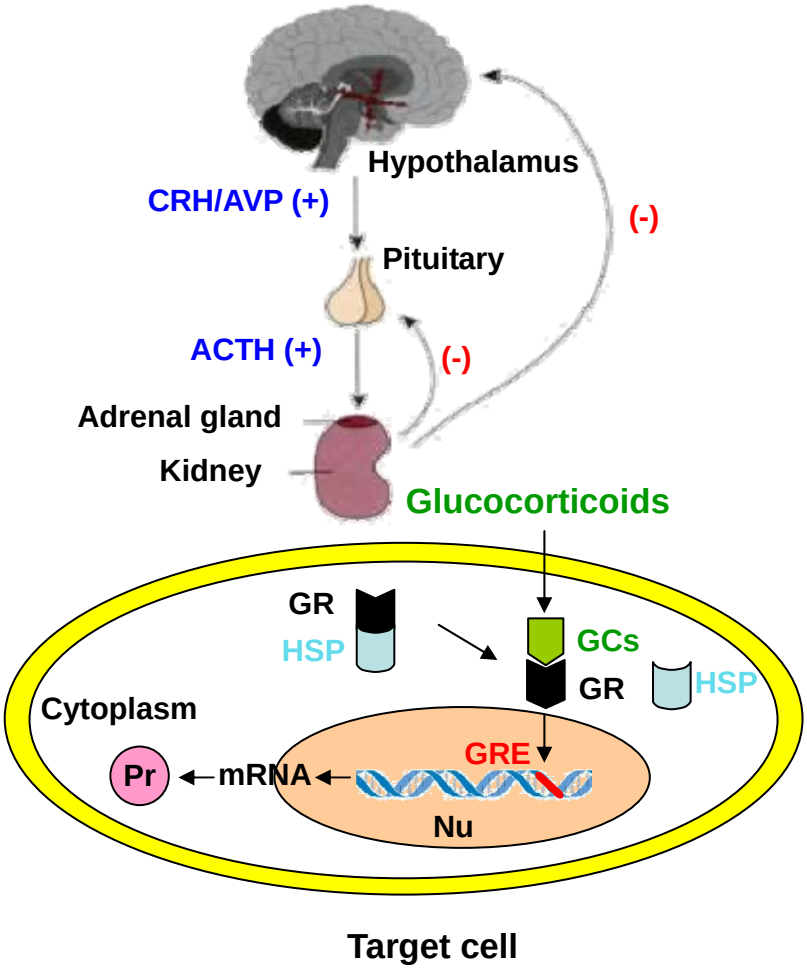


Figure 1-7. Glucocorticoid (GC) secretion is regulated by a negative feed-back loop HPA axis and GCs modulate the target cell gene expression. CRH/AVP is released in the hypothalamus after stimulation, and subsequently stimulates ACTH release from the anterior pituitary which then induces the GC synthesis in the adrenal cortex and release into the circulation. The increased GCs execute feedback inhibition on the HPA axis at both pituitary and hypothalamus, suppressing the synthesis of GC. GCs cross the cell membrane to exert their biological effects by binding to the GR, which exists as a multiprotein complex bound with HSP in the cytoplasm. GC/GR translocates into the nucleus binding to GRE to regulate target gene transcription.

Abbreviations: CRH/AVP, corticotropin-releasing hormone/arginine vasopressin; ACTH, adrenocorticotropin hormone; GCs, glucocorticoids; GR, glucocorticoid receptor; HSP, heart shock protein; GRE, glucocorticoid response element; Nu, nucleus; Pr, protein. Adapted from (Johansson et al. 2007).

1.4.2 Glucocorticoid receptors (GR)

GCs are lipophilic, they can easily cross cell membranes. They then bind to two structurally related intracellular receptors to exert their effects. The glucocorticoid receptor (GR) which is the main receptor in stress and metabolic-regulated tissues is present in almost all cell types, including pancreatic β-cells (Fischer et al. 1990; Matthes et al. 1994). In a few cell types, such as epithelial cells of the kidney (Krozowski et al. 1989), colon (Rafestin-Oblin et al. 1984), mainly those concerned with fluid or electrolyte balance, GCs bind to the mineralocorticoid receptor (MR) (de Kloet 2003). Both receptors act as transcriptional factors that can activate and/or inhibit the transcription of both overlapping and distinct target genes either after binding to DNA or through protein-protein interactions (Datson et al. 2008).

1.4.2.1 Structure of GR

As for other nuclear receptors, GR contains three functional domains. The N-terminal domain consists of sequences responsible for gene activation (Giguere et al. 1986). The DNA binding domain consists of two zinc fingers which are essential for GR binding of target gene promoters through their recognition of specific DNA sequences, glucocorticoid responsive elements (GRE) (Tsai et al. 1988). The ligand binding domain (LBD) is present in the C-terminal which is responsible for hormone binding (Giguere et al. 1986; Warriar et al. 1994), nuclear translocation (Picard and Yamamoto 1987), ligand-dependent transactivation (Webster et al. 1988), and heat shock protein (HSP)-90 binding (Dalman et al. 1991; Pratt 1993).

1.4.2.2 Mechanism of GR action

In the absence of ligand, GR is held in an inactive state in the cytoplasm by its predominant interaction with HSP-90, HSP-70 and immunophilins (FKBP51) (Pratt 1993; Pratt and Dittmar 1998; Pratt et al. 2004). After binding to the ligand, GR undergoes a series of conformational changes that leads to dissociation from the multiprotein complex. This exposes a nuclear localisation signal (Grad and Picard

2007) which activates the GC/GR translocation into the nucleus and subsequent binding to the GRE domain in target genes (Figure 1-7) and regulation of their transcription (Bamberger et al. 1996).

The GR can exert either activating or suppressive effects on target gene transcription. Transactivation occurs when GR binds to the "positive" GRE, and then recruits co-activators to cause chromatin remodelling and facilitation of transcription (Michas et al. 2010). The mechanisms of transrepression of GR are less clear, although it has been proposed that GR binds to a specific "negative" GRE (nGRE), which has a sequence more variable than the canonical GRE (Truss and Beato 1993; de Kloet 2003). GR can either inhibit the target gene transcription directly (Guay et al. 2007) or indirectly by occluding adjacent/overlapping binding sites for the positively acting transcription factors (Schoneveld et al. 2004). The indirect mechanism can also be achieved by protein-protein interaction in which binding to the nGRE is not required (de Kloet 2003; Schoneveld et al. 2004). For example, monomers of GR interact with pro-inflammatory transcription factors, in particular the activator protein 1 (AP-1) and nuclear factor-κB (NF-κB) to prevent pro-inflammatory gene transcription by inhibiting their binding to cognate DNA binding sites (Newton and Holden 2007).

1.4.3 Physiological functions of glucocorticoids and their role in the pancreas

GCs are essential for development, immune responses, energy homeostasis, stress responses, cognition and behaviour. They have wide and profound physiological effects in maintaining homeostasis under physical and emotional stress. The control of GC action within cells is therefore critical. Persistent exposure to supra-physiological GCs causes Cushing's syndrome (Dorn et al. 1995); Deficiency of GCs is found in Addison's disease (Bamberger et al. 1996). Moreover, GC action alteration is typical in the pathogenesis of some diseases states such as Metabolic Syndrome (Seckl and Walker 2004; Reaven 2006).

1.4.3.1 Development

GCs are essential for the organ structure development and tissue maturation (Fowden et al. 1998), especially for lung (Garbrecht et al. 2006) in foetal development. Clinically, GCs are used at late gestation for premature birth to accelerate prenatal lung maturation (Liggins and Howie 1972). However, excess GCs retard foetal growth in both humans (Reinisch et al. 1978) and rodents (Nyirenda and Seckl 1998). Administration of dexamethasone to pregnant rats in the third trimester results in offspring with a low birth weight, which develop hypertension, glucose intolerance and insulin resistance in later life (Benediktsson et al. 1993). Previous studies showed β-cell development is sensitive to GC action. Excessive exposure of the rodent foetus to GCs during the last week of gestation induces impaired glucose tolerance (Lindsay et al. 1996; Nyirenda et al. 1998) and reduction of functional islets (Nyirenda et al. 2009). Both maternal and fetal GCs are increased in undernourished rats, which further results in reduction of foetal β-cell mass and number (Garofano et al. 1997; Garofano et al. 1998). Dexamethasone decreases e15.5 embryonic pancreas progenitor cell differentiation to β-cell and, conversely, increases acinar cell number *in vitro* and *in vivo*, (Gesina et al. 2004).

GR deficient mice die within a few hours after birth from respiratory failure and

atelectasis of the lung which is consistent with GC functions (Cole et al. 1995; Nemati et al. 2008). During mouse embryo development, GR gene expression clearly increases before e13.5 in the gastrointestinal tract, upper respiratory tract, and discrete areas of the central nervous system and in liver. Later, increased expression occurs in lung, muscle, pituitary and thymus (Speirs et al. 2004). In pancreas, GR is expressed at a very low level up to e13·5–e14, increases at e14.5-e16.5 in both islet progenitor cells and the majority of ductal cells, then falls sharply (Speirs et al. 2004). Studies in mice with GR deleted in whole pancreas showed GR was not required for the early induction of pancreatic development and differentiation, although it was necessary for pancreatic morphogenesis during late foetal development through indirect effects (Gesina et al. 2006). A precise GR gene-dosage was important in controlling β-cell expansion during development (Gesina et al. 2006). Moreover, transgenic mice which overexpress GR under the rat insulin I promoter exhibit impaired insulin release which may have developmental origins (Delaunay et al. 1997).

1.4.3.2 Inflammation

As the anti-inflammatory effects of pharmacological levels of GCs are well known, they are commonly used clinically to treat a range of immune/inflammatory disorders, such as allergies, acute inflammation, asthma and rheumatoid arthritis (McEwen et al. 1997; Chapman and Seckl 2008). Endogenous GCs can either potently enhance or suppress immune reactions to shift innate and adaptive immune responses depending on the timing and concentration of exposure (Wilckens 1995; McEwen et al. 1997; Smoak and Cidlowski 2004; Yeager et al. 2004). Thus, GCs exert immunomodulatory effects rather than simply anti-inflammatory effects at sub-pharmacological doses. In innate immunity progression, GCs are essential to reverse the lethal effects of proinflammatory cytokines (IL-1, TNFα) (Bertini et al. 1988) which are secreted by resident macrophages during initiation of the inflammatory response (Topley et al. 1993; Borish and Steinke 2003). Thus, GCs alter leukocyte distribution, differentiation and transcription of numerous genes in GC sensitive leukocytes, monocytes, neutrophils and granulocytes (McEwen et al. 1997) which are recruited by proinflammatory mediators. Moreover, GCs restrain

oedema, increase blood viscosity and haematopoietic differentiation programmes in the response of the innate immune system (McEwen et al. 1997). Notably, GCs also promote adaptive immunity by regulating Th1/Th2 balance (Kovalovsky et al. 2000). T lymphocytes (T-cells) are classified into two broad subsets. The pathogenic cellular immune response is mediated by Th1 cells, a subset of T cells characterized by secretion of IL-2 and INFγ which induce macrophages and $CD8^+$ cells to secrete proinflammatory cytokines. Alternately, the protective humoral immune response is mediated by Th2, a subset of T cells that synthesize IL-4 and IL-10 which are considered as anti-inflammatory effectors (Rabinovitch 1994). GCs suppress action of the Th1 cellular immune response and may help to explain the shift toward the Th2 humoral immune response (Blotta et al. 1997; Ramirez 1998). GCs also both increase macrophage secretion of IL-10 (Ramirez et al. 1996) and induce the development of T regulatory cells (Barrat et al. 2002), thus suppressing immune responses, and promoting the resolution of inflammation. The anti-inflammatory and immunosuppressive action of GCs is mainly due to the inhibition of several transcription factors (TF) activity such as NF-κB, AP-1 and STAT proteins, through direct interaction with GR (Cippitelli et al. 1995; Scheinman et al. 1995; Liu et al. 2001).

Inflammation is a key feature of the autoimmune disease T1D. Further, over the last decade, a close link between inflammation and T2D has been established (Wellen and Hotamisligil 2005). T2D rodent models have an increased number of immune cells both in the circulation (Kolb and Mandrup-Poulsen 2005; Wellen and Hotamisligil 2005) and within key metabolic tissues such as adipose (Weisberg et al. 2003) and pancreatic islets (Ehses et al. 2007). Considering GCs have diabetogenic properties, promoting insulin resistance, and other side effects (Detailed in 1.4.3.3), they are not an optimal clinical treatment for long-term suppression of inflammation. However, notably, GCs were recently used for preconditioning islet preparations prior to islet transplantation to reduce later potential immunorejection responses through careful modulation of the dose (Lund et al. 2008).

1.4.3.3 Metabolism

GCs are essential for maintaining glucose homeostasis under normal conditions and contribute to dysregulated glucose homeostasis in pathological conditions (McMahon et al. 1988; Friedman et al. 1996; Andrews and Walker 1999; Jacobson et al. 2006). In the prolonged fasted state the glucose levels fall and active GCs are released into the circulation to promote hepatic glucose production. In contrast, when the glucose level increases after food intake, GCs assist glycogen synthesis (a form of energy storage) by activating the glycogen synthesis pathway in the presence of insulin (Stalmans and Laloux 1979; Ruzzin and Jensen 2005). However, chronic elevation of GCs through exogenous administration or excessive production of endogenous GCs (e.g. Cushings syndrome) is associated with dyslipidaemia, hypertension, glucose intolerance and insulin resistance (Landy et al. 1988; Chrousos 1995; Reynolds et al. 2001; Andrews et al. 2002) which contribute to the development of T2D and cardiovascular disease.

GCs regulate glucose metabolism in several tissues and organs, the mechanism involves increasing gluconeogenesis in the liver, mobilising lipids and glycerol from adipose tissue and amino acids from muscle as well as decreasing glucose uptake in muscle (reviewed in (Vegiopoulos and Herzig 2007)). In general, GCs are described as diabetogenic hormones for their potent effect to stimulate glucose production and induce insulin resistance in both humans and rodents, although notably most studies have used pharmacological doses of the potent sythetic GR agonist dexamethasone (Tappy et al. 1994; Nicod et al. 2003; Buren et al. 2008; Rafacho et al. 2008).

1.4.3.3.1 Glucocorticoids and liver

In liver, GCs promote gluconeogenesis resulting in the generation of glucose from non-carbohydrate carbon substrates such as amino acids and glycerol. They address this mainly by activating two critical enzymes: phosphoenol pyruvate carboxykinase (PEPCK) (Hanson and Reshef 1997) and glucose-6-phosphatase (G6Pase) (Garland 1986). PEPCK is a rate limiting enzyme in gluconeogenesis. The PEPCK promoter contains two positive GC response elements (GRE) and three accessory factor

elements (Hanson and Reshef 1997). G6Pase catalyses the terminal step in gluconeogenesis and its promoter contains two potential GREs (Lin et al. 1998) which enable GCs to elevate its activity (Nordlie and Arion 1965; Garland 1988).

1.4.3.3.2 Glucocorticoids and adipose tissue

In adipose tissue, GCs regulate lipid metabolism according to the level of the GR, which is fat depot selective, and the prevailing nutritional state, fasting or fed. GCs increase lipolysis through activating hormone sensitive lipase and inhibiting lipoprotein lipase in peripheral fat in the fasted state (e.g. subcutaneous, gluteal and thigh) (Ong et al. 1992; Slavin et al. 1994; Mattsson and Olsson 2007). The released FFAs are essential for oxidation in other tissues, particularly muscle, to generate energy. The released glycerol is a substrate for hepatic gluconeogenesis. Glucocorticoids also promote pre-adipocyte differentiation, induce lipogenic activity, and increase hypertrophy in central fat (e.g. abdominal, visceral adiposity) (Gaillard et al. 1991; Samra et al. 1998; Masuzaki et al. 2001; Morton and Seckl 2008). Consistent with these effects, Cushing's syndrome patients show a marked redistribution of body fat, with fat and muscle wasting in subcutaneous and limbs, but accumulation in the depots of the abdomen, the nape of the neck and face where GR levels are higher.

1.4.3.3.3 Glucocorticoids and muscle

In skeletal muscle, GCs modulate protein metabolism by promoting protein degradation, inducing the mobilization of amino acids, increasing amino acid export and inhibiting protein synthesis (Vegiopoulos and Herzig 2007). The metabolised amino acids serve as substrates for liver gluconeogenesis in times of prolonged fast, or with GC excess. Furthermore, GCs suppress glucose oxidation by direct inhibition of the glucose transport system (Olefsky 1975). Peripheral glucose uptake depends on the cell membrane translocation of the GLUT4 transporter, which is expressed mainly in skeletal muscle. GCs suppress GLUT4 expression and translocation to the cell membrane from an internal location which suppress glucose uptake (Horner et al. 1987; Oda et al. 1995; Weinstein et al. 1995; Coderre et al. 1996; Dimitriadis et al.

1997).

1.4.3.3.4 Glucocorticoids and pancreas

Numerous studies have examined GCs effects on pancreatic islet glucose-stimulated insulin secretion (GSIS). However, no consensus has emerged due to the divergent results found across different species and methodologies. *In vitro*, GCs inhibit GSIS from perfused rat pancreas (Billaudel and Sutter 1979; Barseghian et al. 1982) isolated rodent islets (Billaudel et al. 1984; Pierluissi et al. 1986; Wang et al. 1994; Gremlich et al. 1997; Lambillotte et al. 1997; Zawalich et al. 2006; Sandberg and Borg 2007; Swali et al. 2008), and in a mouse model of obesity (Chan and Lejeune 1992; Davani et al. 2000; Ortsater et al. 2005). The inhibitory effects were only present with high physiological to supraphysiological GC exposure (10 and 100 nmol/l dexamethasone, a potent synthetic GC or 50 and 100 nmol/l 11-dehydrocorticosterone (11DHC), the inactive GC in rodent) and led to both suppression of insulin release (Davani et al. 2000; Jeong et al. 2001; Swali et al. 2008) and insulin biosynthesis (Jeong et al. 2001). Evidence for GC-mediated increases in GSIS has been found in normal C57BL/6J mouse islets (Brunstedt and Nielsen 1981; Hult et al. 2009). (Table 1-2)

However, the direct effects of GCs on islet function is difficult to address *in vivo*, because GCs have diverse effects on other key metabolic tissues (see 1.4.3.3). Thus because GRs are widely distributed in many cells; the multiple influences of GCs in the whole body makes the results hard to interpret. Notably, GCs may enhance vagal stimulation of insulin secretion by their central action (Stubbs and York 1991; Angelini et al. 2010), adding further complexity to the overall *in vivo* effects of GC excess.

In vivo, GC treatment induces peripheral insulin resistance in humans (Beard et al. 1984; Tappy et al. 1994; Nicod et al. 2003; Ahren 2008) and rats (Severino et al. 2002; Holness et al. 2005; Buren et al. 2008; Rafacho et al. 2008), which elicits a secondary compensatory response from the pancreatic β-cell to promote insulin secretion by increasing β-cell mass and/or function (Beard et al. 1984; Karlsson et al.

2001; Choi et al. 2006; Rafacho et al. 2008). Accordingly, the isolated islets from dexamethasone treated rats display an increase of GSIS due to increased mitochondrial function, Ca^{2+} signaling, PKC activity, and changes in exocytotic capacity (Rafacho et al. 2010). It is difficult to unravel whether some, none or all of these changes are due to direct effects of the GCs on β-cell function. (Table 1-2)

In contrast leptin deficient obese $Lep^{ob/ob}$ and normal mice treated with GCs in comparable dose to the rats (Rafacho et al. 2008) exhibit reduced GSIS *in vivo* (Khan et al. 1992; Ling et al. 1998). Mice with GR overexpression which is under the control of the rat insulin I promoter also exhibit impaired insulin release (Delaunay et al. 1997; Ling et al. 1998; Davani et al. 2004). The mechanism by which increased GC sensitivity (higher β-cell GR) directly inhibit GSIS in mice remains unclear but likely involves regulatory factors of the glucose metabolism pathway, such as increased glucose cycling through G6Pase activity (Khan et al. 1992; Khan et al. 1995; Ling et al. 1998), decreased GLUT2 transporter stability (Gremlich et al. 1997), and augmented α_2-adrenergic receptor level which inhibits insulin secretion (Davani et al. 2004). (Table 1-2)

For human, intravenous administration of cortisol increases the first phase of β-cell function (Vila et al. 2010) and oral administration of dexamethasone increases insulin secretion (Abdelmannan et al. 2010). Overall, it has been difficult to discern the primary effects on β-cell function from those arising from peripheral insulin resistance-induced compensatory responses. (Table 1-2)

Table 1-2. GCs effects on pancreatic islet glucose-stimulated insulin secretion

In vivo/vitro	Species	Source	Type of GCs	Dose	Method	Effects	Reference
In vitro	Rat	Perfused pancreas	Cortisone	1mM	Repeated pulse administration	Inhibition	(Barseghian et al. 1982)
	Rat (male albino)	Perfused pancreas	Corticosterone	0.02, 0.2 or 20mg/l	Pre-incubated for 1hr. 4.2mM and 16.7mM glucose	Inhibition. Only 2nd phase disturbed.	(Billaudel and Sutter 1979)
		Isolated islets				Inhibition. Inhibitory effects rapid than pancreas.	
	Rat (male albino)	Isolated islets	Corticosterone	0.6μM	Pre-incubation for 40-44mins	Inhibition by reduce Ca^{2+} influx.	(Billaudel et al. 1984)
	Rat (Sprague-Dawley)	Isolated islets	Dexamethasone, prednisolone, hydrocortisone	6.3μM	Pre-incubation for 2hrs, 2mM and 20mM glucose	Inhibit GSIS. Delayed 1st phase, reduced 2nd phase.	(Pierluissi et al. 1986)
			Aldosterone, hydrocortisone	8nM			
	Rat (male Sprague-Dawley)	Isolated islets	Dexamethasone	1μM	Pre-incubation for 48hrs, 2.8mM and 16.7mM glucose	Inhibit GSIS by reduce GLUT2	(Gremlich et al. 1997)
	Rat	Isolated islets	Dexamethasone	1μM	Pre-incubation for 3hrs, 15mM glucose	Inhibit both 1st phase and 2nd phase	(Zawalich et al. 2006)
	Rat (Sprague-Dawley)	Isolated islets	Dexamethasone	1, 10, 100nM	Pre-incubation for 6hrs, 3.3, 16.7mM glucose	Inhibit in dose dependent	(Jeong et al. 2001)
				1nM	Pre-incubation for 1hr	No effect	

				10, 100nM		Inhibition	
	Mouse (male NMRI)	Isolated islets	Dexamethasone	1 μM	Pre-incubation for 5hrs, 15mM glucose	No acute, even transient, inhibitory effect	(Lambillotte et al. 1997)
					Pre-incubation for 18hrs, 3mM and 15mM glucose	Inhibition in both glucose	
				20nM	Pre-incubation for 18hrs, 15mM glucose	Inhibition ~50%	
				250nM		Inhibition ~75%	
	Mouse (male NMRI)	Isolated islets	Corticosterone	1, 10, 100 μM	3.3 or 5.5mM glucose	Inhibition. Concentration dependent	(Sandberg and Borg 2007)
	Mouse (male C57BL/6J)	Isolated islets	Dexamethasone	0.5, 5, 50nM	3 or 20mM glucose	Inhibition.	(Swali et al. 2008)
			Corticosterone	50nM			
	Rat (fa/-)	Isolated islets	Dexamethasone	1nM	Pre-incubation for 24, 48, 72hrs, 27.5mM glucose	Inhibition.	(Chan and Lejeune 1992)
	Rat (fa/fa)			10nM	Pre-incubation for 72hrs, 27.5mM glucose		
	Mouse ($Lep^{ob/ob}$)	Isolated islets	11-DHC	50, 500nM	3.3, 8.3, 16.7mM glucose	Inhibition in a dose-dependent	(Davani et al. 2000)
	Mouse ($Lep^{ob/ob}$)	Isolated islets	11-DHC	5nM	Pre-incubation for	No effect	(Ortsater et al.

				50, 500 nM	48hrs, 3,11mM glucose	Inhibition	2005)
	Mouse (C57BL/6J)			50nM	Pre-incubation for 48hrs	No impact on GSIS	
	Mouse (C57BL/6J)	Isolated islets	Hydrocortisone	1nM	Islet cultured for 1 to 3weeks.	Increase	(Brunstedt and Nielsen 1981)
	Mouse (C57BL/6J)	Isolated islets	Corticosterone	200nM	Pre-incubation for 18hrs, 3,11mM glucose	Increase 1st and 2nd phase during 120hrs	(Hult et al. 2009)
In vivo	Rat	Isolated islets	Dexamethasone	4mg/kg	ip. For 10 days, 2.8mM glucose and 20mM glucose	Inhibition	(Wang et al. 1994)
	Rat (female Sprague-Dawley)	Isolated islets	Dexamethasone	2mg/kg	ip. For 12 days, 3.3mM and 8.3mM glucose	Increase GSIS.	(Karlsson et al. 2001)
					ip. For 12 days, 0-20mM glucose	Increase insulin secretion at 1.8mM to 11.1mM glucose	
					Islets cultured overnight.	Increase insulin secretion at 1.8 to 3.3mM glucose, inhibit at 11.1 to 20mM glucose	
	Rat (male Wistar)	Isolated islets	Dexamethasone	0.1, 0.5mg/kg	ip. for 5 days, 2.8-22mM glucose	Increase GSIS, counteract metabolic disorder	(Rafacho et al. 2008)
				1mg/kg		Increase GSIS, can not counteract metabolic disorder	
	Rat (male Wistar)	Isolated islets	Dexamethasone	1mg/kg/day	ip. for 5 days, 2.8, 11.1mM glucose	Increase insulin secretion in both 2.8 and 11.1mM glucose	(Rafacho et al. 2010)

	Rat (male Wistar)	Isolated islets	Dexamethasone	1mg/kg/day	ip. For 5 days. 8.3mM glucose	Increase	(Angelini et al. 2010)
	Mouse ($Lep^{ob/ob}$)	Isolated islets	Dexamethasone	25μg/day	ip. For 1 or 2 days, 5.5 and 16.7mM glucose	Inhibit GSIS	(Khan et al. 1992)
	Mouse (C57BL/6J, RIP-GR)	Isolated islets	Dexamethasone	100μg/ml	ip. For 3 days, 5.5 and 16.7mM glucose	Inhibit GSIS	(Ling et al. 1998)
	Human (men)	Circulation plasma	Dexamethasone	6mg/day.	3 mg twice daily for 2 days,	Increase	(Beard et al. 1984)
	Human (men)	Circulation plasma	Hydrocortisone (cortisol)	0.6mg/kg	iv. 4 mins before administrate 330mg/kg glucose	Increase 1^{st} phase insulin secretion (15mins)	(Vila et al. 2010)
	Human	Circulation plasma	Dexamethasone	2, 4, 8mg	Oral, 24 hrs before OGTT	Increase insulin secretion. Small effects of 2, 4mg. significant effects in 8mg.	(Abdelmannan et al. 2010)

1.5 11β-hydroxysteroid dehydrogenases (11β-HSDs)

Besides GR density, the primary determinants of physiological GC action depends on the intracellular concentration of the ligand which is regulated by the activity of the HPA axis, by the level of the corticosteroid binding globulin, and by the activity of the intracellular 11β-hydroxysteroid dehydrogenase enzymes. The intracellular 11β-HSD activity was discovered by Amelung and colleagues (1953). 11β-HSD activity is found in a broad range of cells and tissues (reviewed in Seckl and Walker 2004). There are two isozymes of 11β-hydroxysteroid dehydrogenase, 11β-HSD1 and 11β-HSD2, that catalyze the interconversion of hormonally active GC (cortisol in humans and corticosterone in rodents) and inactive GC (cortisone in humans and 11DHC in rodents) to regulate intracellular GCs levels (reviewed in Tomlinson et al. 2004). 11β-HSD1 acts as a bi-directional (the reductase usually being predominant) enzyme and it is largely expressed in the tissue and organs associated with high GR expression, such as liver, lung, adipose tissue, and brain (Moisan et al. 1990; Tannin et al. 1991; Ricketts et al. 1998; Jamieson et al. 2000; Brereton et al. 2001; Dzyakanchuk et al. 2009). In contrast, 11β-HSD2 functions solely as a dehydrogenase and is expressed mainly in kidney and other mineralocorticoid target tissues where it prevents inappropriate activation of GCs (Cook et al. 1988; van Uum et al. 2004). Notably, MR and GR have indistinguishable affinities for active GCs and as such the 11β-HSD enzymes fullfil a critical pre-receptor ligand access function in these tissues (Seckl and Walker 2004).

1.5.1 11β-hydroxysteroid dehydrogenases type 1 (11β-HSD1)

The 11β-HSD1 gene in rat contains an 861bp open reading frame encoding a protein of 288 amino acids (Lakshmi and Monder 1988). Analysis of the mouse cDNA shows extensive conservation of nucleotide (91%) and amino acid (86%) sequences with rat 11β-HSD1 (Rajan et al. 1995). Fetal mouse 11β-HSD1 mRNA expression is first observed at e15 in lung and liver, and its expression increases markedly from

this time (Speirs et al. 2004).

11β-HSD1 is an NADP(H)-dependent enzyme which is a major regulator of GC metabolism in mammals (Agarwal and Auchus 2005). In tissue homogenates (Lakshmi and Monder 1988) and some transfected cells (Agarwal et al. 1989; Bujalska et al. 1997; Atanasov et al. 2004; Dzyakanchuk et al. 2009) 11β-HSD1 exerts a bi-directional function. However, in intact cells and *in vivo*, 11β-HSD1 mainly acts as a reductase (Low et al. 1994; Jamieson et al. 1995; Kotelevtsev et al. 1997; Jamieson et al. 2000). 11β-HSD1 is an integral membrane protein of the endoplasmic reticulum (ER) with its N-terminus anchor in the ER membrane and with the C-terminus inside the ER lumen (Ozols 1993; Mziaut et al. 1999; Dzyakanchuk et al. 2009). 11β-HSD1 activity highly depends on the ratio of NADPH/$NADP^+$ within the ER lumen (Atanasov et al. 2004; Banhegyi et al. 2004; Bujalska et al. 2005; Dzyakanchuk et al. 2009). Because the ER membrane is relatively impermeable to pyridine nucleotides, the NADPH/$NADP^+$ ratio within the ER is regulated by other enzymes, key among which is hexose-6-phosphate dehydrogenase (H6PDH) which is untethered and resides in the ER lumen (Ozols 1993). The H6PDH is the microsomal counterpart of the cytosolic glucose-6-phosphate dehydrogenase (G6PDH). $NADP^+$ and G6P are the two essential substrates for H6PDH to generate NADPH by catalyzing the first two steps of the endoluminal pentose-phosphate pathway. Generation of NADPH thereby drives 11β-HSD1 reductase activity (Lavery et al. 2006). Unlike $NADP^+$, G6P is transported into the ER lumen by the glucose-6-phosphate transporter (G6PT) (Chou et al. 2002). Deficiency of the G6PT in liver results in a loss of 11β-HSD1 reductase activity (Walker et al. 2007). Numerous *in vitro* studies have demonstrated a key link between 11β-HSD1 reductase activity and H6PDH (Atanasov et al. 2004; Banhegyi et al. 2004; Bujalska et al. 2005; McCormick et al. 2006). Critically, H6PDH knock-out mice exhibit a switch in 11β-HSD1 activity from reductase to dehydrogenase in liver, strongly suggesting the enzyme is the key co-factor provider for 11β-HSD1 *in vivo* (Lavery et al. 2006). Furthermore, H6PDH expression is stimulated by dexamethasone in both adipocytes *in vitro* and rat adipose tissue *in vivo* (Balachandran et al. 2008), suggesting regulation of H6PDH as well as

11β-HSD1 is an important determinant of intracellular GC action. (Figure 1-8)

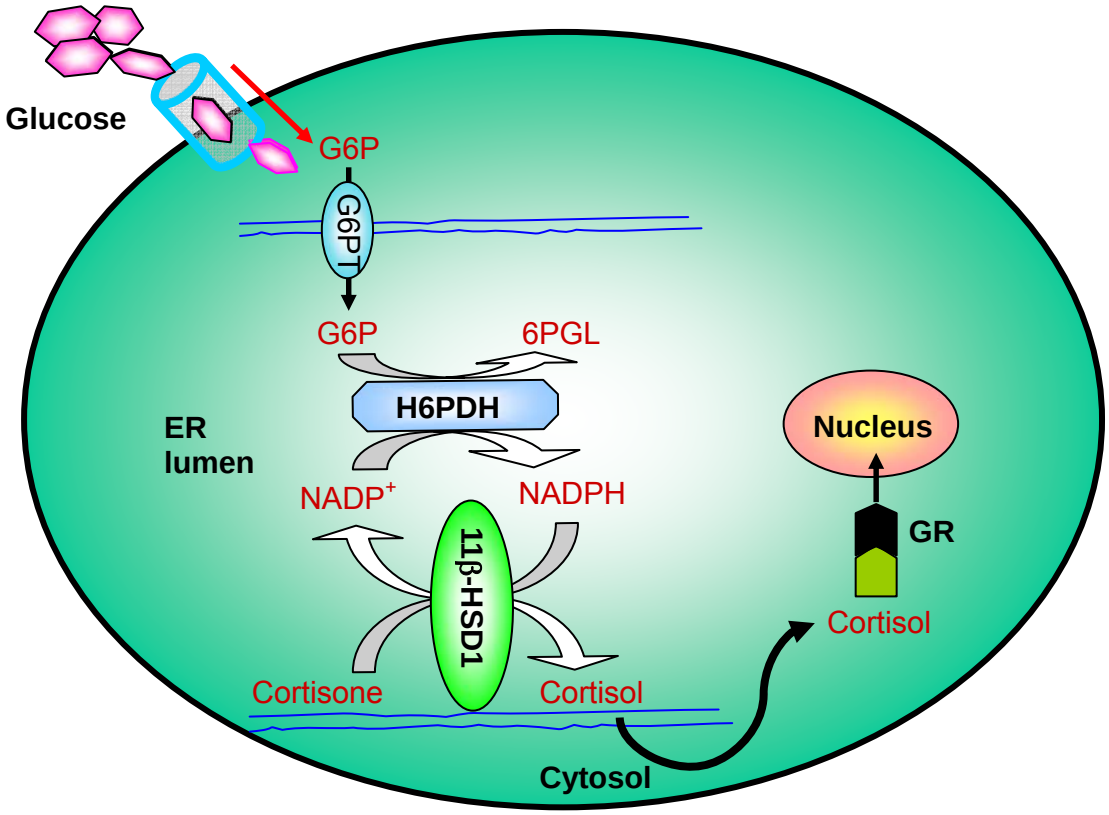


Figure 1-8. The interaction between 11β-HSD1 and H6PDH. Glucose is taken up by the cell and metabolised to generate G6P which enters the ER lumen through G6PT. G6P can be utilized by H6PDH in the presence of $NADP^+$ to provide NADPH which is a cofactor that permits reductase (cortisone to cortisol) activity of 11β-HSD1. The active cortisol subsequently binds to GR to exert its effects in the nucleus.

Abbreviations: ER, endoplasmic reticulum, H6PDH, hexose-6-phosphate dehydrogenase, NADP, Nicotinamide adenine dinucleotide, NADPH, Nicotinamide adenine dinucleotide phosphate, G6P, glucose-6-phosphate; G6PT, Glucose-6-phosphate transporter; 6PG, 6-phosphogluconolactonate (adapted from Tomlinson et al. 2004; Zielinska et al. 2011).

1.5.2 The promoter of the 11β-HSD1 gene

Different promoter usage and alternate splicing mechanisms make alternate 11β-HSD1 mRNA transcripts in distinct tissues. Although two different cDNAs were cloned from mouse liver (Oppermann et al. 1995; Rajan et al. 1995), that encode identical proteins, the cDNA sequences differ in the 5′ untranslated region (UTR), suggesting alternate transcription starts. Previous studies (Bruley et al. 2006) have used 5′ rapid amplification of cDNA ends (RACE) to identify an alternate promoter, P1, which is located 23kb upstream to the commonly used promoter, P2. Both promoters are expressed in liver, lung, adipose tissue, and brain. However, P1 (encoding exon 1A) predominats in lung and P2 (encoding exon 1B) predominates in liver, adipose tissue, and brain. P1 and P2 derived transcripts encode the same protein, 11β-HSD1 A. Importantly, this implies distinct regulatory mechanisms may control 11β-HSD1 transcription in a tissue-specific manner. A further promoter, P3 has been described in kidney which initiates the transcription at another site in rat; 264 bp further 5′ as well as within the first intron (Moisan et al. 1992). The latter start site transcribed an 11β-HSD1 mRNA encoding a nonfunctional protein 11β-HSD1 B (Mercer et al. 1993; Obeid et al. 1993). (Figure 1-9)

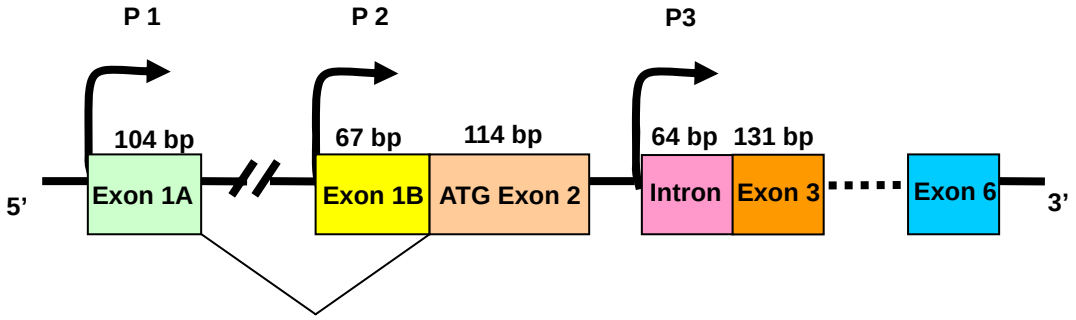


Figure 1-9. The promoter region of the 11β-HSD 1 gene. Organisation of the rodent 11β-HSD1 gene, P1, encoding Exon 1A; P2 encoding Exon 1B; Exon 1A is 23kb upstream of the Exon 1B; P3, encoding an intron. When transcription begins from promoter 1, Exon 1A is spliced with the Exon 2; from promoter 2, the Exon 1B is spliced with the Exon 2, however translation begins from the same ATG, so they encode the same protein 11β-HSD1 A. In kidney, transcription begins at another promoter close to Exon 3, encoding the non-functional protein 11β-HSD1 B. Adapted from (Bruley et al. 2006).

1.5.3 Factors regulating 11β-HSD1

The regulation of 11β-HSD1 transcription and activity is highly tissue-specific and influenced potently by hormones such as GCs, insulin, as well as cytokines such as proinflammatory IL-1 and TNF-α (Jamieson et al. 2000; Tomlinson et al. 2004). GCs temporally regulate 11β-HSD1 expression and activity in rat liver and hippocampus *in vivo* (Moisan et al. 1990; Jamieson et al. 2000) and dose-dependently increase 11β-HSD1 expression in human skeletal muscle (Whorwood et al. 2002) GCs generally act to stimulate 11β-HSD1 in skin fibroblasts (Hammami and Siiteri 1991), testis (Nwe et al. 2000), human coronary artery smooth muscle cells (Michas et al. 2010), preadipocytes (Kim et al. 2007), and hepatocytes (Liu et al. 1996; Voice et al. 1996). However, in adipocytes, GCs down-regulate 11β-HSD1, at least *in vitro* (Napolitano et al. 1998; Balachandran et al. 2008) whilst up-regulating 11β-HSD1 *in vivo* (Livingstone et al. 2000; Balachandran et al. 2008). Insulin inhibits 11β-HSD1 expression and/or activity in many cells (Moisan et al. 1990; Hammami and Siiteri 1991; Liu et al. 1996; Jamieson et al. 1999; Nwe et al. 2000; Sandeep et al. 2005; Kim et al. 2007). In contrast, insulin can stimulate

11β-HSD1 expression and activity in adipocytes *in vivo* and *in vitro* (Wake et al. 2006; Westerbacka et al. 2006; Balachandran et al. 2008). Taken together, both GCs and insulin regulate 11β-HSD1 possibly in a concentration-dependent and cell-specific manner. Proinflammatory IL-1 and TNFα increase 11β-HSD1 mRNA levels in several cell types (Escher et al. 1997; Tetsuka et al. 1999; Cai et al. 2001; Cooper et al. 2001; Tomlinson et al. 2001; Yong et al. 2002). The TNFα effect is attenuated by insulin in human adipose stromal cells (Handoko et al. 2000). Growth hormone (GH) (Liu et al. 1997; Moore et al. 1999), liver X receptor (LXR) and peroxisome-proliferator-activated receptor-γ (PPARγ) (Berger et al. 2001; Stulnig et al. 2002) inhibit 11β-HSD1 expression and activity. Moreover, high fat diet decreased 11β-HSD1 activity in adipose tissue without effects in liver though the mechanisms for this remain unknown (Morton et al. 2004).

Members of the CAAT/enhancer-binding protein (C/EBP) family are the major transcription factors regulating 11β-HSD1 (McKnight et al. 1989; Darlington et al. 1995; Croniger et al. 1998; Poli 1998). The 11β-HSD1 P2 promoter contains 10 C/EBP binding sites between 2812 and 176 (Williams et al. 2000). Among the C/EBP isoforms expressed in liver and adipose C/EBPα, which is important in energy metabolism (McKnight et al. 1989) and haematopoietic lineage determination (Iwasaki et al. 2006), acts as a potent activator of the 11β-HSD1 P2 promoter in hepatocytes both *in vivo* and *in vitro* (Williams et al. 2000; Bruley et al. 2006). *In vitro*, several cell lines, such as preadipocytes (Napolitano et al. 1998; Gout et al. 2006; Arai et al. 2007), lung epithelial cells (Sai et al. 2008) and skin fibroblasts (Hammami and Siiteri 1991), 11β-HSD1 activity is elevated through C/EBPβ binding to the P2 promoter. Furthermore, 11β-HSD1 expression is increased by TNFα in hepatoma cells (Ignatova et al. 2009), and IL-1 in human foetal lung fibroblasts (Yang et al. 2009) through the P2 promoter and C/EBPβ. C/EBPβ plays a role in inflammatory and innate immune functions, so it can be a link between inflammation and metabolism (Poli 1998). In addition, GCs are important regulators of C/EBP transcription, markedly inducing C/EBPβ and C/EBPδ mRNAs (Matsuno et al. 1996; Breed et al. 1997; Gotoh et al. 1997) and with differing temporal and

tissue-specific effects on C/EBPα mRNA (MacDougald et al. 1994; Ramos et al. 1996). However, the regulation of P1 and P3 remains poorly understood. The P1 promoter is not regulated by C/EBPα in transfected cells (Bruley et al. 2006), and is expressed independently of C/EBPα in lung.

1.5.4 11β-HSD1 in obesity and lessons from transgenic mouse models

GCs play an important role in the development of obesity and metabolic disease in rodents as evidenced by the correction of all metabolic abnormalities with adrenalectomy (ADX) and re-instatement of the aberrant phenotype with GC replacement in the ADX animals (Shimomura et al. 1987; Dallman et al. 2004). However, circulating GC levels are normal or even low in common or idiopathic human obesity (Pasquali et al. 1999) whereas tissue 11β-HSD1 activity, particularly adipose tissue, is elevated (Bujalska et al. 1997; Wake et al. 2003; Wake et al. 2006). This led to the concept that amplification of tissue GCs may explain the similarities between certain aspects of excess GC action (e.g. the visceral obesity, type 2 diabetes and increased cardiovascular risk) found in Cushings syndrome with that found in common obesity.

1.5.4.1 11β-HSD1 in the adipose tissue

11β-HSD1 is highly expressed in fat and its expression and activity increases with differentiation from pre-adipocytes to mature adipocytes (Napolitano et al. 1998). 11β-HSD1 expression and/or activity increased in adipose tissue of obese humans (Rask et al. 2001; Rask et al. 2002; Lindsay et al. 2003; Wake et al. 2003; Kannisto et al. 2004; Sandeep et al. 2005; Goedecke et al. 2006; Michailidou et al. 2007) and rodents (Livingstone et al. 2000; Masuzaki et al. 2001; Morton et al. 2004). In rodents, 11β-HSD1 is particularly increased in visceral fat (Livingstone et al. 2000; Morton et al. 2004), whereas, it increased in the subcutaneous fat in humans (Sandeep et al. 2005). 11β-HSD1 overexpression specifically in adipose tissue under the aP2 promoter (aP2-HSD1) achieved a level of 11β-HSD1 activity that was

comparable (2-3 fold higher than lean) to that found in obese humans. Crucially, as with idiopathic human obesity corticosterone levels in the blood were normal but corticosterone was higher in adipose tissue and the portal circulation draining from the visceral fat (Masuzaki et al. 2001). aP2-HSD1 mice developed glucose intolerance, insulin resistance, dyslipidemia, leptin resistance and a "metabolic syndrome" (Masuzaki et al. 2001) as well as hypertension (Masuzaki et al. 2003), making a causal link between elevated adipose 11β-HSD1 and metabolic syndrome.

1.5.4.2 11β-HSD1 in the liver

In contrast to adipose tissue, in liver, the organ with the highest level of 11β-HSD1 expression (>10-fold compared to adipose tissue (Tannin et al. 1991)) 11β-HSD1 expression is decreased in obese humans (Stewart et al. 1999; Rask et al. 2001; Rask et al. 2002; Sandeep et al. 2005) and rodents (Livingstone et al. 2000; Liu et al. 2003). However, the aP2-HSD1 transgenic mice display a high level of corticosterone and FFAs in the hepatic portal vein (Masuzaki et al. 2001), which suggests overexpression of 11β-HSD1 in visceral adipose tissue could cause a detrimental delivery of elevated GCs to liver, at least in mice. The liver-specific overexpression of 11β-HSD1 was generated by utilizing the ApoE promoter (ApoE-HSD1) (Paterson et al. 2004) to investigate the effects of excess hepatic GCs on metabolic syndrome. ApoE-HSD1 mice exhibited modest insulin resistance, increased liver fat content, and dyslipidaemia in the absence of glucose intolerance, obesity or changes of adipose distribution (Paterson et al. 2004). Along with an improvement in liver insulin sensitivity in humans after treatment with the 11β-HSD1 inhibitor carbenoxolone (Walker et al. 1995; Rosenstock et al. 2010), these data indicated that elevated hepatic 11β-HSD1 activity, as is found in certain rare conditions such as myotonic dystrophy (Johansson et al. 2001), makes a pathogenic contribution to metabolic disease (Liu et al. 2005; Morton et al. 2005).

1.5.4.3 11β-HSD1 in the skeletal muscle

Although skeletal muscle is an important target for GC-induced insulin resistance (describe in 1.4.3.3), the expression of 11β-HSD1 is relatively low in skeletal muscle

in mice (Itoh et al. 2004). In contrast, 11β-HSD1 is expressed in vascular smooth muscle (Walker et al. 1992). Human skeletal muscle cells express 11β-HSD1, and its activity is down regulated by insulin *in vitro* (Whorwood et al. 2001). 11β-HSD1 is increased in skeletal muscle of a diabetic rat model, but decreased in diabetic subjects (Zhang et al. 2009). The role of skeletal muscle 11β-HSD1 remains to be clarified, although recent work has demonstrated that 11β-HSD1 inhibitors have direct beneficial effects in rodent skeletal muscle (Morgan et al. 2009).

1.5.5 The 11β-HSD1 knock out mouse model

Absence of regeneration of active corticosterone after complete disruption of the 11β-HSD1 gene in tissues of 11β-HSD1 knockout mice (11β-HSD1$^{-/-}$) confirmed that 11β-HSD1 was a reductase in most intact cells, and that only the 11β-reductase was capable of locally reactivating 11-dehydrocorticosterone to corticosterone in mice (Kotelevtsev et al. 1997). To compensate the low production of active GCs, 11β-HSD1$^{-/-}$ mice exhibit adrenal hypertrophy, higher secretion of corticosterone, hyper-responsiveness to acute restraint stress, and low sensitivity to the negative feedback on HPA axis (Kotelevtsev et al. 1997; Harris et al. 2001).

11β-HSD1$^{-/-}$ mice are resistant to diet-induced metabolic syndrome (visceral obesity, insulin resistance, type 2 diabetes, and dyslipidemia), which resembles Cushing's Syndrome of plasma GC excess. Thus 11β-HSD1$^{-/-}$ mice had increased hepatic and adipocyte insulin sensitivity (Morton et al. 2001; Morton et al. 2004). 11β-HSD1$^{-/-}$ mice on a high fat diet gain less weight and preferentially accumulated peripheral rather than visceral fat mass, in part due to an increase PPARγ expression and activation in visceral fat (Morton et al. 2001; Morton et al. 2004; Wamil et al. 2011). Moreover, 11β-HSD1$^{-/-}$ mice exhibit improved lipid and lipoprotein profiles associated with increased HDL cholesterol and reduced VLDL triglyceride levels (Morton et al. 2001; Morton et al. 2004). 11β-HSD1$^{-/-}$ mice also exhibit improved glucose tolerance, in part through lower fasting induction of PEPCK and G6Pase, thereby decreasing gluconeogenesis in liver (Kotelevtsev et al. 1997). 11β-HSD1$^{-/-}$ mice are insulin-sensitized, particularly in adipose tissue, and show a reduction of

leptin, resistin and TNFα, but elevation of adiponection and PPARγ (Morton et al. 2001; Morton et al. 2004; Densmore et al. 2006). Moreover, 11β-HSD1$^{-/-}$ mice display a transient hyperphagia (Morton et al. 2004; Densmore et al. 2006), enhanced angiogenesis after myocardial infarction (Small et al. 2005), are resistant to cognitive decline in old age (Sooy et al. 2010) and have an exaggerated inflammatory response and altered inflammatory resolution (Gilmour et al. 2006; Zhang and Daynes 2007). Taken together these studies show that intracellular amplification of GC action by 11β-HSD1 has a profound effect on many of the fundamental processes ascribed to GC (see 1.4.3) clearly emphasising the importance of tissue-level regulation of GC action, often in the face of or independently of HPA control.

1.5.6 11β-HSD1 inhibitors

Given the protective effects of 11β-HSD1 deficiency in metabolic disease, a strategy to develop a therapeutic inhibitor of 11β-HSD1 has been considered as a treatment for metabolic syndrome and T2D (Boyle 2008; Morton and Seckl 2008; Morton 2010; Tomlinson et al. 2010). Carbenoxolone, a compound purified from liquorice is a non-selective 11β-HSD1 inhibitor with major efficacy in liver, poor efficacy within adipose tissue, but with capacity to improve insulin sensitivity in humans and rats (Walker et al. 1995; Livingstone et al. 2000). However, the weight of evidence suggested that dysregulation of 11β-HSD1 clearly occurs in the adipose tissue in obesity. Thus, compounds that would be efficacious across a number of tissues were sought. Arylsulfonamidothiazoles (BVT.14225 and BVT.2733) were the first reported selective 11β-HSD1 inhibitors and they enhanced insulin action in liver, as well as lowering blood glucose concentrations in diabetic and obese mice (Alberts et al. 2002; Barf et al. 2002; Alberts et al. 2003). To date, a number of other 11β-HSD1 inhibitors are under development for potential clinical purposes (Alberts et al. 2003; Hermanowski-Vosatka et al. 2005; Webster et al. 2007; Bujalska et al. 2008). Recently, the first data using an 11β-HSD1 inhibitor for the clinical treatment of T2D has been reported (Rosenstock et al. 2010). Patients treated with INCB13739 added to ongoing metformin monotherapy had significantly reduced hemoglobin A1C, fasting glucose, total cholesterol, LDL, cholesterol, and triglycerides, while the basal

cortisol level was unchanged. The prospects for therapeutic inhibition of 11β-HSD1 are therefore good, although the downstream effects of these inhibitors in tissues where elevated 11β-HSD1 may play a beneficial functional role (e.g. macrophage inflammation resolution) remains to be clarified.

1.5.7 11β-HSD1 in the pancreas and diabetes

11β-HSD1 is expressed in rodent (Davani et al. 2000; Duplomb et al. 2004; Ortsater et al. 2005; Swali et al. 2008) and human pancreatic islets (Davani et al. 2000). Recently 11β-HSD1 expression was found to be highest in the α cells suggesting a paracrine modulation on insulin secretion exists (Swali et al. 2008). 11β-HSD1 expression is higher in pancreatic islets of obese/diabetic rodents and is more sensitive to 11-dehydrocorticosterone (11DHC) mediated suppression of GSIS, an effect reversed by 11β-HSD1 inhibitors (Davani et al. 2000; Duplomb et al. 2004; Ortsater et al. 2005; Swali et al. 2008). Curiously, however, exposure of normal islets to 200nM corticosterone produced a stimulation of first phase GSIS, a finding that was not interpreted in the publication, although the suppressive effect of corticosterone on second phase GSIS was discussed (Hult et al. 2009). Furthermore, the high glucose and lipid levels typical of metabolic disease were assessed for their potential regulatory effects on islet 11β-HSD1 *in vitro*, but no clear effect on 11β-HSD1 mRNA levels on isolated islets of prediabetic ZDF rats was found (Duplomb et al. 2004). Thus, the increased GC action mediated by elevation of 11β-HSD1 expression may modulate the onset of diabetes by amplifying the inhibitory effect of GCs in β-cell. However, according to inconsistent and controversial effects of GCs on islet GSIS (details in 1.4.3.3), it is equally possible that the altered β-cell 11β-HSD1 could be secondary to the β-cell failure, or even a mechanism invoked to protect the β-cell from further damage. Therefore, overall, the physiological role of islet 11β-HSD1 and its impact upon the etiology of diabetes is unclear.

1.6 Hypothesis and Aims

Hypothesis

This thesis was based upon the over-arching hypothesis that:

Elevated β-cell 11β-HSD1 levels promote β-cell failure and type 2 Diabetes.

Aims

The overall aim of this thesis was to investigate the role and regulation of 11β-HSD1 in the pancreatic β-cells. To accomplish this goal, investigations were carried out by generating a β-cell-specific 11β-HSD1 overexpression model in mice. The specific aims were:

1. To investigate what regulates 11β-HSD1 in pancreatic islets, including:
    i) Which activity directionality of 11β-HSD1 is predominant in islets;
    ii) Which promoter controls 11β-HSD1 expression in islets;
    iii) Which factors regulate islet 11β-HSD1 activity in diabetes.
2. To determine the effects of β-cell-specific overexpression of 11β-HSD1 on β-cell failure in type 2 diabetes.
3. To determine the effects of β-cell-specific overexpression of 11β-HSD1 on β-cell failure in type 1 (streptozotocin-induced) diabetes.

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Animals and animal care

Diabetes-susceptible C57BL/KsJ mice were obtained from Jackson laboratory (Jackson laboratory, USA) at 4 to 6 weeks of age, and were subsequently bred in the biomedical research facility (Little France, University of Edinburgh). Mice were housed in a temperature controlled room maintained with a 12hour light/dark cycle (7am-7pm light, 7pm-7am dark). Mice were given standard chow (RM, Special Diets Services) and autoclaved water *ad libitum.* Conditions and diets changed for specific experiments (described below). All studies were approved by the local ethical committee of the University of Edinburgh and were performed within the guidelines of the United Kingdom Animals Scientific Procedures Act, 1986 and under the auspices of the approved project license PPL 60/3962.

2.1.1 Creation of a β-cell-specific 11β-HSD1 overexpressing mice

A model of β-cell 11β-HSD1 overexpression was created using a β-cell specific promoter (Mouse insulin-I (Hara et al. 2003), a gift from Dr. Mark Magnusson, Vanderbilt University, USA). The sequence of the mouse insulin promoter (8.3kb) was cloned upstream of the rat 11β-HSD1 cDNA (1.265bp) in a pGEM11Zf (+) vector (constructed by Lynne Ramage) before being purified and micro-injected into blastocysts from C57BL/KsJ (performed by Genetic Intervention and Screening Technologies Biomedical Research Resources, University of Edinburgh) and implanted in pseudo-pregnant females. The creation of the transgenic mice model had been performed by Nicholas M. Morton, Sophie Turban and Lynne Ramage. Control C57BL/KsJ mice were bred in house. Only male C57BL/KsJ mice were used for the experiments in this thesis.

2.2 Experimental diets

Mice were fed a hypercaloric (high-fat) diet (58 kcal% fat w/sucrose Surwit Diet, Research diet, New Brunswick, USA) for 12 weeks to induce glucose intolerance, and hypocaloric (low-fat) diet (11 kcal% fat w/cornstarch Surwit Diet, Research diet) to be control.

2.3 Glucose tolerance test (GTT)

The GTT was used to determine how quickly glucose was cleared from the blood after glucose administration. This method gives a crude measure of insulin sensitivity (glucose disposal) and β-cell function (where insulin evolution curves act as a surrogate for β-cell responsiveness to glucose load).

2.3.1 Oral glucose tolerance test (oGTT)/ intraperitoneal glucose tolerance test (ipGTT)

Mice were housed individually several days before the experiment to allow them to equilibrate to their environment and reduce undue stress/HPA activation. Prior to glucose administration, mice underwent a 6 hour fast to achieve a 'baseline' blood glucose level. Blood glucose levels were measured in a drop of blood after venesection of the tail vein using a handheld glucometer (OneTouch Ultra, LifeScan, USA). Further blood samples were collected in a Microvette CB 300 (Sarstedt, Leicester, UK) at time zero (T_0) to determine baseline plasma insulin levels. A bolus of glucose solution (2g glucose/kg bodyweight using 25% (w/v) D-glucose in 0.9% NaCl solution for ipGTT, in dH_2O for oGTT) was either injected into the intraperitoneal cavity or delivered into the stomach by a gavage needle (20-gauge, 38 mm long curved, with a 21/4 mm ball end; Able Scientific, Canning Vale, Western Australia, Australia). Blood was collected at 1, 3, 5, 10, 15, 30, 60, and 120 minutes after glucose administration for glucose and insulin analysis.

2.3.2 Intravenous “long” glucose tolerance test (lGTT)

Due to the variability in absorption in the ipGTT and, to a lesser extent the oGTT, we developed an intravenous glucose tolerance test (IvGTT, lGTT). The lGTT closely reports the critical first (1 to 3 minutes) and second phase of insulin secretion after glucose challenge. This improves the accuracy of our interpretation compared to intraperitoneal GTT and oral GTT which can exhibit marked variation in time of glucose absorption, especially when there are large anatomical variations in body fat; e.g. in obese compared to lean animals. The lGTT also used a continuous infusion of a higher dose of glucose than ipGTT or oGTT in order to cause sustained maximal insulin release (5g/kg BW for one hour) to help distinguish β-cell function from peripheral glucose disposal effects. In the absence of euglycaemic/hyperinsulinaemic clamps, this gave us improved insight into β-cell capacity/function.

2.3.2.1 Jugular vein cannulation:

Each mouse was anaesthetized by injection of 1mg/ml Medetomidine (Domitor) and 100mg/ml Ketamine (Vetalar), (0.1ml/10 grams body weight, ip), and placed on its back on a pre-warmed hot pad. With the aid of a dissecting microscope (Zeiss Semi 2000), a vertical incision was made above the jugular vein. The jugular vein was found and isolated. Four pieces (approximately 2cm each) of 5-0 braided silk suture material were placed under the vein. The upper ligature was tied to occlude the vein. The centre ligatures were left loose. The catheter was prepared by attaching a 10 cm length of Micro-Renathane tubing 025" O.D. x .012" I.D (Sandown Scientific, Hampton, UK) to a 25G needle. A needle was attached to a 1ml syringe filled with a saline heparin mix (heparin: saline: 1:10). The bent needle tip was then used to cause a small puncture in the jugular vein and the catheter was subsequently fed into the vessel until it reached the pectoral muscle. Any occlusions were checked by aspirating the syringe catheter assembly gently; blood from the vein flows freely in and out of the catheter when a good insertion is achieved. Ligatures were tied to firmly secure the catheter, and any excess thread was trimmed off. One small drop of surgical glue (3M™ Vetbond™ Tissue Adhesive, 3M, US) was placed to make sure the catheter remained stable. The catheter was taken off from the needle and closed

at the needle end by tightly knotting. The mouse was turned on its stomach and a small incision was made through the skin around the back of the neck. Thin blunt end forceps were fed subcutaneously toward the right shoulder to create a path to exteriorize the catheter. The excess catheter was folded and stored under the skin of the back to prevent it being damaged by the mouse (biting) upon recovery. All wounds were then closed using mouse surgery clips (A-75， Perfect, Bruneau, France). Reversal agent 5mg/ml antipamezole (antisedan, 0.05ml/10g) was administered, Vetegesic analgesic was administered as a diluted solution in sterile water (1:10, 0.05ml/30g), and 1ml saline was injected intraperitoneally to counteract any dehydration from the hotplate. Mice were put in a 30 °C warm-box overnight and observed for 2 days for full recovery. Surgery routinely lasted less than 30 minutes per mouse.

2.3.2.2 Infusion procedure:

After a minimum 48h of surgical recovery the mice were fasted for 4 hours on the morning of the experiment. An infusion pump CMA/102 microdialysis pump (CMA Microdialysis; Phymep) was used to infuse either saline or glucose solution (25% (w/v) D-glucose dilute in 0.9% NaCl solution) into the jugular vein and hence directly in a carefully time-controlled fashion into the blood circulation. The saline was loaded in a 1ml syringe attached a 25G needle and run through P10 tubing (Jencons pls, Leighton Buzzard, UK) to a 25G needle connector, which was connected to the catheter. A small portion of the mouse tail tip was clipped and blood glucose level was measured by OneTouch Ultra Blood Glucose Monitoring System (Johnson and Johnson Company, Lifescan, Bucks, UK). Initially, a constant low flow rate (0.2µl/min) of saline was infused for 2 hours (to allow any stress effects on basal glucose to subside) blood glucose was sampled before starting the infusion pump (time 0). The glucose solution was infused with a flow rate of 10µl/min/30g bodyweight. Blood glucose was measured and blood samples were taken for insulin measurement with a Microvette CB 300 (Sarstedt, Leicester, UK) at 1, 3, 5, 10, 15, 30, 60 minutes. The glucose bolus was stopped at 60 minutes and final glycemia was measured at 120 minutes.

2.4 Analysis of plasma corticosterone

Age-matched (10 to 20 weeks) animals were housed singly several days prior to blood sampling. Mice were given standard chow diet or high fat diet (58% calories as fat for 12 weeks, as described before in 2.1.2) *ad libitum.* Blood samples were obtained from a tail venesection within 1 minute of disturbing the cage to avoid confounding stress-mediated elevation of corticosterone. Samples were taken during the diurnal nadir of the HPA cycle (between 8:30 and 9:00am).

2.5 Streptozotocin (STZ) experiments

Streptozotocin (STZ) is a naturally occurring chemical from a strain of the soil microbe Streptomyces achromogenes (Malcolm 1962) and has been widely used in animal experiments to induce type I like diabetes (Mansford and Opie 1968; Wang and Gleichmann 1998).

2.5.1 High-dose STZ experiment

Age matched mice were given a single bolus i.p. injection of either 10mM citrate buffer (PH4.5) or STZ (180mg/kg body weight, dissolved in 10mM citrate buffer, Sigma-Aldrich). Blood glucose was monitored by sampling a single drop of fresh blood from a single tail venesection (tail nick; re-opened gently with a damp paper towel) everyday at around 2pm. Mice were monitored until being killed at either 3 or 10 days after STZ treatment. Trunk blood samples were collected on the last day and pancreata were harvested and either frozen or fixed in 10% formalin (Sigma-Aldrich, Company Ltd, Dorset, UK) for further investigation.

2.5.2 Multiple low-dose STZ experiment

The mice were treated either with i.p. injection of saline or STZ (40mg/kg body weight) dissolved in saline for 5 consecutive days. Induction of diabetes was assessed by taking tail blood glucose measurements at day 0, 4, 12, and 15.

2.6 Measurement of key factors in plasma

Blood samples were placed immediately on ice and then centrifuged at 2000rpm for 10mins. The plasma was separated and stored at -80°C until assayed.

2.6.1 Plasma glucose measurement

Enzymatic glucose assay: A standard curve (33.3, 22.2, 11.1, 5.55 and 2.8mM) was made from a stock solution at the reference standard of 11.1mM (Thermotrace, Leeds, UK) by adding different volumes (3µl, 2µl, 1µl, 0.5µl, 0.25µl) using 96-well plates. Glucose concentration was measured by adding 2µl of standards or samples in 10µl water, then supplementing with 200µl of glucose infinity reagent (Thermotrace) into each well. The plate was incubated at 37°C for 3 min and glucose concentration was calculated based on the absorbance of the standards and samples measured by a Spectrophotometer at a wavelength of 340nm.

Glucose meter blood glucose measurement: blood glucose level was measured in a single drop of blood from a tail tip or venesection using OneTouch Ultra Blood Glucose Monitoring System (Johnson and Johnson Company, Lifescan, Bucks, UK)

2.6.2 Plasma insulin measurement by ELISA

Plasma insulin level was measured using a Mouse insulin ELISA kit (Crystal chem, Downers Grove, US) according to the manufacturer's protocol. The standard curve (12.8, 6.4, 3.2, 1.6, 0.8, 0.4, 0.2, 0.1, 0ng/ml insulin) was made by diluting stock standard solution of 25.6ng/ml mouse insulin provided in the kit. 95µl of sample diluents were dispensed in each well of a 96 well plate, and 5µl of sample or standard curves was added and incubated for 2 hours at 4°C. Contents were aspirated and washed 5 times with appropriate buffer solution (300µl of washing buffer per well), then 100µl per well of anti-insulin enzyme conjugate was added and incubated for 30mins at room temperature. Well contents were aspirated and the wells were washed 7 times using 300µl of washing buffer per well. 100µl per well of enzyme substrate solution was added and the solution was allowed to react for 40mins at

room temperature without exposure to light. The reaction was stopped by adding 100µl per well of enzyme reaction stop solution. The insulin concentration of the samples was calculated with reference to the standard curve by measuring the absorbance within 30minutes using a platereader (Spectrophotometer) at A_{450}. Background was accounted for by subtracting A_{630} values. Sample blanks and interassay controls were routinely used to compare between experiments, although all results presented were from self-contained experiments in this thesis (i.e. relative values are compared between groups within a given experiment).

2.6.3 Insulin measurement by RIA

Insulin released into the medium from isolated pancreatic islets (large experimental groups dictated a more practical choice of assay for the *in vitro* work) was measured using an rat insulin RIA kit (Linco Research, Milipore Missouri, USA) according to the manufacturer's protocol. On the first day, 100µL of hydrated ^{125}I-Insulin was added in "total activity" tubes, 200 µL of assay buffer plus hydrated ^{125}I-Insulin was added to the "Non-Specific Binding" (NSB) tubes and 100 µL of assay buffer plus 100µL of rat anti-insulin antibody plus hydrated ^{125}I-Insulin to "Reference tubes". Duplicated 100 µL of "Standards" and "Quality Controls", then 100 µL of each sample were combined with 100 µL of hydrated ^{125}I-Insulin along with 100 µL of rat anti-insulin antibody. Samples were then vortexed, covered, and incubated overnight at 4°C. On day 2, 1 ml of cold (4°C) precipitating reagent was added to all tubes except Total Count tubes and samples were vortexed and incubated for 20 minutes at 4°C. All tubes except Total Count tubes were centrifuged at 4°C for 20mins. The supernatant of all tubes except Total Count tubes were immediately decanted, drained for at least 15-60 seconds, and excess liquid was blotted from the lip of the tubes. All tubes were counted in a gamma counter for 1 min. (This work has been done by Sophie Turban and Lynne Ramage, Edinburgh)

2.6.4 Plasma corticosterone measurement by radioimmunoassay

Plasma corticosterone was measured by RIA using a ^{3}H-corticosterone tracer and a polyclonal anti-corticosterone antibody (a gift from Dr C.J. Kenyon, Edinburgh).

Plasma was diluted in borate buffer 1:10 and denatured at 75°C for 30mins. 2µl of ^{3}H-corticosterone (62.2 Ci/mmol; Perkin-Elmer Life Science, Boston, USA) was added into 9ml of borate buffer, and then antibody was added for a final dilution $1:10^{4}$. 50µl of the mixture dilution buffer and 20µl standard or samples were added to each assay well of a 96-wells plate, gently swirled/mixed and then incubated at room temperature for at least one hour. 50µl SPA (Scintillation proximity assay beads, GE Healthcare) was added to each well, samples were inverted continuously for several times and then incubated at room temperature for 24hrs. A scintillation (1450 Liquid Scintillation Counter, Wallac, Boston, USA) was used to measure the radioactive counts (cpm, counts per minute). The antigen-antibody reaction was competitive meaning that the more radioactive counts the less corticosterone was present in the wells.

2.7 Isolation of islets from mouse pancreas

Mice were anesthetized with sodium pentobarbital (30mg/kg). Pancreas was inflated with 3ml of a 1mg/ml collagenase XI (Sigma Aldrich) disolved in Hank's balanced salt solution (HBSS, Lonza, Slough, UK) via the bile-pancreatic duct to aid rapid digestion the surrounding exocrine pancreas (Salehi et al. 1996). Whole pancreas was removed, and further digested in collagenase for 20mins at 37°C. Digested pancreata were then incubated on ice for 15min with 1v/v HBSS containing 10% fetal bovine serum (FBS, Lonza). The debris-containing supernatant was discarded and the cell suspension was washed 2 times with HBSS containing 10% FBS and then centrifuged at 1000rpm for 3min to gentle pellet the islets between washes. Islets were hand picked manually under a binocular microscope at 10× magnification in a blackened Petri dish.

2.8 Genotyping of transgenic mice

2.8.1 DNA extraction from tail

Genomic DNA was extracted from the tail of each mouse to determine its genotype. The tail was incubated in 600µl tail buffer (see table 2-3) containing 35µl 10mg/ml proteinase K (Sigma-Aldrich). The tails were gently rotated at 55°C in an oven overnight. The next day and 20µl of 20µg/ml RNase A (Sigma-Aldrich) was added into each tube, and incubated at 37°C in a hotblock for one hour. 37.5µl of 2M β-mercaptoethanol (Sigma-Aldrich) and 600µl phenol (Fisher Scientific, Leicestershire, UK) was added into each tube. The tubes were rotated for 15mins and then centrifuged at 12,000g for 2mins. The aqueous phase was removed into a fresh tube (using cut-off pipette tips to avoid genomic DNA shearing). 300µl of phenol and 300µl of chloroform: isoamyl alcohol (24:1, Sigma-Aldrich) was added, and the tubes were rotated for 5mins then centrifuged at 12,000 for 2mins. The aqueous phase was transferred to a fresh tube. 600µl chloroform: isoamyl alcohol (24:1) was added, and the tubes were rotated for 5mins then centrifuged at 12,000g for 2mins. The aqueous phase was transferred to a fresh tube. 600µl isopropanol (Fisher Scientific) was added, and the tubes were inverted several times to precipitate the DNA. DNA was pelleted by centrifuging at 12,000g, for 2mins. The supernatant was removed and discarded. The pellets were resolubilised after brief drying in 200µl TE buffer by warming at 37°C for 30mins (or leaving at 4°C overnight). 100µl of 6M ammonium acetate which removed the free nucleotide and protein plus 600µl isopropanol were added to purify the DNA. Samples were centrifuged at 12,000g for 2mins to pellet the DNA. The supernatant was removed carefully. The pelleted DNA was washed with 180µl 70% ethanol (VWR, Leicestershire, UK), and centrifuged at high speed for 2mins, the ethanol was removed and air dried briefly. 30µl TE buffer was added to resolubilise the DNA and it was stored at 4°C.

2.8.2 Polymerase Chain Reaction (PCR)

The PCR reactions (50µl) contained 0.2µg DNA, 1.25U GoTaq (Promega, Southampton, UK), and 30pmol of each primer (Manufactured by Invitrogen, Renfrew, UK). Primers for genotyping were MIP-HSD1: 5'-GGA ACT GTG AAA CAG TCC AAG G-3', and MIP-HSD1: 5'-TTT GCT GGC CCC AGT GAC AAG CTT T-3'. (Figure 2-1)

PCR program was 98°C for 3 mins, then 40 cycles of 90°C for 45secs (denaturation), 60°C for 30secs (annealing), 72°C for 90secs (extension), then finally 72°C for 5mins and 4°C for 10mins.

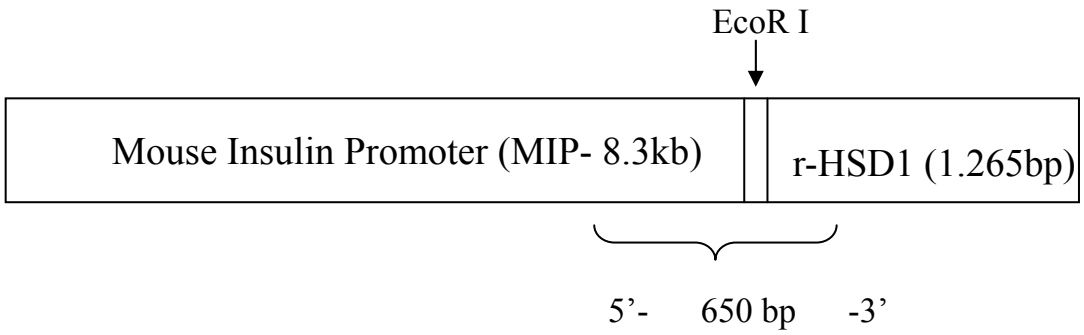


Figure 2-1. PCR site of the MIP-r11β-HSD1 transgene. The product of PCR is 650bp long including part of MIP gene, part of the r11β-HSD1 gene, and a restriction enzyme EcoR I site (designed by Sophie Turban).

2.8.2.1 Restriction enzyme digestion of cDNA to identify the presence of the transgene (MIP-HSD1) insert

20µl of cDNA from the PCR amplification mouse tail DNA was incubated with the specific MIP primers containing a restriction site for EcoR I along with 1µl EcoR I, 2µl buffer (H) (Promega), in a total reaction volume of 25µl and the reaction was incubated at 37°C for 2 hours to identify the existence of the specific transgene.

2.8.2.2 Agarose gel electrophoresis

2% agarose gels were prepared for analysing the PCR DNA fragment restriction digestion patterns by electrophoresis. Agarose (Lonza, Slough, UK) was dissolved in 0.5x TBE buffer by boiling the solution in a microwave oven. Ethidium bromide (final concentration 0.2µg/ml, Sigma-Aldrich) was added to the melted agarose and cast in horizontal gel trays. Set gels were submerged in 0.5x TBE in standard gel electrophoresis tanks and samples was loaded into wells. DNA was separated by electrophoresis under constant voltage conditions of 140V. Sizes of DNA fragments were determined by comparison with a 100bp DNA ladder (Invitrogen) run in parallel. Resolved DNA fragments were visualized and pictured by placing agarose gels on a UV transilluminator using a wavelength of 254nm.

2.8.3 Southern Blotting

2.8.3.1 DNA digestion

Genomic DNAs (10-30µg) from mouse tail was restriction enzyme digested with 4µl Afl III (5000 units/ml, Biolab, Herts, UK) restriction enzyme which has two sites present in MIP-HSD1 transgene (Figure 2-2), 4µl Buffer 3, 4µl BSA (1mg/ml), and proper dH_2O to make a final volume of 40µl. Samples were incubated overnight in at 37°C.

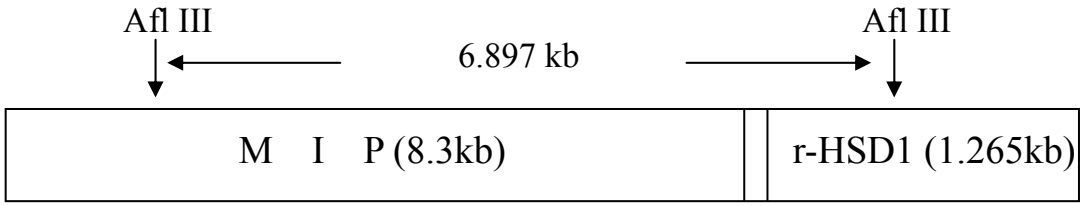


Figure 2-2. Afl III enzyme restriction sites of the MIP-HSD1 transgene. There are two digestion sites for Afl III in MIP transgene. One site is in the MIP (2861) and the other one was at the end of r-HSD1 cDNA (9758). The length between two Afl III sites was 6.897kb which included most of the MIP-HSD1transgene.

2.8.3.2 Gel electrophoresis of restriction digested genomic DNA

After digestion was completed, DNA samples were run on a 0.7% agarose gel; agarose was dissolved in 1× TAE buffer. Samples were loaded with 4µl DNA loading buffer and run in 1× TAE buffer at 40V for 40mins, then at 100V until the bromophenol blue dye arrived around a ¾ of the total length of the gel. The gel was removed and cut to a suitable size for transfer.

2.8.3.3 Transfer of DNA to nylon membranes

The DNA was depurinated by exposing the gel to 0.15M HCl for 10 mins; then 0.5M NaOH, 1.5M NaCl for 45mins. This was neutralized by submerging the gel in 1.5M Tris, 1.5M NaCl, 1mM EDTA (PH 7.2) for 30 mins, and again in fresh buffer for 15 mins. The transfer blot was set up by laying 3 pieces of 3M paper, soaked in 10× SSC on top of an inverted gel tray. The gel was placed on top and a sized piece of Zeta-Probe GT Genomic Tested Blotting membrane (Bio-Rad Laboratories Ltd. Hertfordshire, UK) was placed directly onto the gel. This was followed by 2 pieces of pre-wetted 3M paper. A stack (about 10cm thick) of folded blotting paper was placed on the top to initiate capillary transfer and the DNA was transferred for 24 hours.

2.8.3.4 Pre-hybridization

After transferring for 24 hours, the membrane was rinsed briefly in water to remove excess salt and then baked at 80°C for 2 hours to fix the DNA. The membrane was then pre-hybridized with 5× Denhard's (Sigma-Aldrich) reagent containing 100ug/ml denatured salmon testes DNA (Sigma-Aldrich) (boiled for 15 minutes at 100^{o}C and rapidly cooled on ice or added directly to the hybridisation vessel), 0.5% SDS, and 6×SSC, and incubated at 65°C for at least 6 hours.

2.8.3.5 Hybridization

2.5µl of probe DNA (10ng/µl, rat 11β-HSD1, 1.265kb) and TE buffer 42.5µl were mixed and heated at 95°C for 5 min then put on ice for 5 min. The mixture and 5µl of

α-^{32}P CTP (3000 Ci/mmol, GE Healthcare, Buckinghamshire, UK) were added into Amersham Ready to go DNA Labelling Beads (-dCTP, GE Healthcare) mixed well and incubated at 37°C for 15 min. A Nick column (Sephadex, G-50 DNA Grade, GE Healthcare) was equilibrated with TE buffer. The mixture was pipetted directly onto the column. 400µl of TE buffer was applied, and this first eluate fraction (unincorporated) was discarded. Another 400µl TE buffer was added to elute the probe and the labeled probe was collected in another tube. The denatured and chilled probe was added to 20ml of hybridization buffer (0.5% SDS; 6×SSC) at 65°C. Pre-hybridisation buffer was removed and hybridisation buffer was added and the membrane was incubated overnight at 65°C.

2.8.3.6 Washes and Exposure

After overnight hybridization, the probe mixture was discarded and the membrane was washed in the hybridisation bottle with 20 ml of washing solution in the following steps, 15 min twice with 2× SSC, 0.1% SDS; 65°C, 15 min twice with 1× SSC, 0.1% SDS. The membrane was wrapped in saran wrap and placed against autoradiographic film (MS, Kodak, US) and exposed at -80°C for a week before being developed in a dark room. Alternatively the membrane was exposed to a phosphor imager ^{32}P-sensitive screen overnight and read on a radioimaging detector (FLA-2000, Fujifilm) and analysed by quantitative phosphorimager software Aida / 2D densitometry (Aida 344, Raytek Scientific, Sheffield, UK)

2.9 11β-HSD1 Activity Assay

Thin Layer Chromatography (TLC) is used to separate different compounds in a mixture. Here it was used to detect levels of either 11-dehydrocorticosterone (11DHC, A compound) or corticosterone (B compound) newly made by 11β-HSD1 using tritiated tracer A or tritiated B. The relative amount of both tritiated compounds within the same sample reveals the rate of conversion from one to the other which reflects the activity of 11β-HSD1. Thus reductase activity is measured in cells by treatment with tritiated 11DHC and dehydrogenase activity with tritiated

corticosterone. The compounds are identified by their migration with respect to pure cold 11DHC or B added to the samples before loading TLC plates. Cold compounds can be visualised under UV light and their migration pattern marked and cross-referenced with the tritium signal from the samples.

Routinely, 100 Isolated islets were cultured in 24 wells plates and incubated in 500µl RPMI 1640 (Lonza) with 10% FBS, supplemented with 10nM [^{3}H] tracer A or B and 200nM cold A or B in order to measure the reductase or dehydrogenase activity respectively. Different concentrations of cytokines and factors according to experiment design (see below) were added to the culture medium. Islets were then incubated at 37°C for 24 hours.

11DHC and corticosterone were extracted from the medium using ten volumes of ethyl acetate. The ethyl acetate fraction was collected and evaporated using both air flow and a heating plate at 70°C, leaving steroid residues in the tubes. Samples were resuspened in 40µl 100% ethanol, and spotted in 5µl aliquots at 2cm from the bottom line of TLC plates (TLC Silica Gel60 F254, Merk, Germany). Samples were placed 1.5cm apart. The plates were placed in a pre-equilibrated chamber containing 92ml chloroform and 8ml ethanol as a mobile phase and allowed to run for one hour. Steroids were separated by capillary action, the fast migrating spot was identified with the cold standard 11DHC, and the slower migrating spot was corticosterone. Plates were dried and then exposed to a phosphorimager tritium detection film (BAS-TR2040, Fujifilm) in a cassette for at least 48 hours. The conversion of steroids was calculated after scanning analysis using a radioimaging detector (see 2.6.2) that measured ^{3}H signal with densitometry using quantitative phosphorimager software Aida / 2D densitometry (see 2.6.2). Conversion was calculated with the densitometry data from the signal of the newly generated ^{3}H steroid as a percentage of the whole steroid in the sample. Activity was interpreted as accurate if it was within a linear range with respect to the sample protein concentration and conversion (usually below 40%). With isolated islets, the activity assay applied in this thesis did not go beyond the linear range.

2.10 Insulin release from isolated islets

Mouse islets were isolated and prepared in batches of 10 islets, chosen to reflect a similar mean size (100μm) between genotypes. Islets were placed on a Millicell 8.0 μm insert (Milipore, Billerica, USA) within a 24 well plate and treatments were performed in triplicate. Islets were equilibrated in 500μl KREBS solution (0.1% BSA) with 2.8mM glucose at 37°C in a 5% CO_2 incubator. After 2 hours, KREBS buffer was replaced with 500μL of fresh KREBS solution with 2.8mM glucose every 10min for 30min followed by 500μl KREBS solution with a stimulatory glucose concentration at 25mM every 5 min for 30min and by 500μL KREBS solution with 2.8mM glucose every 10 for 30min to demonstrate the full capacity of the islets to return to basal levels of insulin production. Media were collected and frozen for further analysis of insulin secretion. The insulin concentration was measured by RIA kit (Linco research, Missouri, USA) according to the manufacturer's protocol as described in 2.6.3 and this work was performed by Sophie Turban and Lynne Ramage.

2.11 Insulin content in whole pancreas

Pancreas were taken and weighed, 2ml lysis buffer was added and each pancreas was homogenized using a motorized homogenizer (IKA). 100ul of homogenate was then diluted in 2ml acidified ethanol (1.5% HCl in 70% ethanol) overnight at -20°C. The next day samples were centrifuged at 2000rpm for 15mins at 4°C. The aqueous solution was transferred to new tubes and neutralized by the addition of 100μl 1M Tris (PH7.5). Insulin content was measured by RIA kit (see 2.6.3) and corrected for the appropriate dilution factor.

2.12 Protein content in whole pancreas

Bio-Rad Dye Reagent Concentrate (Bio-Rad Laboratories, Britain) was diluted 1:4 in

distilled water. 2mg/ml BSA was diluted to make a standard curve (2, 1, 0.5, 0.25, 0.125, and 0.0625mg/ml). 200µl of diluted Bio-Rad Dye Reagent and 5µl standard curve or diluted samples (containing ~1-2mg of protein, samples from plasma was diluted 1 in 10^4) were added in to 96 wells plates and reacted at room temperature for 15mins. Protein content was assessed from the absorbance of the sample with respect to the standard curve at 570nm using a plate reader spectrophotometer.

2.13 RNA Extraction

2.13.1 Extraction of RNA from whole pancreas

RNA extraction was extracted with the TRIzol (Invitrogen, Paisley, UK) method. Briefly 1ml TRIzol was added to pancreas (~100-200mg) and the tissue were homogenised using a motorized homogenizer (see 2.11). Homogenates were incubated at room temperature for 5min before the addition of 0.2v/v of chloroform (Sigma, Gillingham, UK). The tubes were briefly shaken to mix and then incubated at room temperature for 3min. Samples were then centrifuged at 12,000g for 15min at 4°C. This produces a lower red phenol-containing phase (containing proteins), an interphase (containing DNA and denatured proteins), and an upper aqueous phase (containing RNA) which was transferred to a fresh eppendorf tube. Isopropanol (0.5v/v, Sigma) was then added to precipitate the RNA. Samples were incubated at room temperature for 10min and then centrifuged at 12,000g for 10min at 4°C. The supernatant was discarded and the RNA pellet was then washed with 1ml 75% ethanol. Samples were inverted and centrifuged at 12,000g for 5min at 4°C. The RNA pellets were allowed to air dry at room temperature for 5-10min. Pellets were resuspended in 15µl DEPC water (0.1% diethylpyrocarbonate, Invitrogen), RNA concentration was determined using a GeneQuant spectrophotometer (Pharmacia Biotech, Sweden).

2.13.2 Extraction of RNA from pancreas by RNeasy mini column

Pancreas (~10-20mg) was homogenised in 0.5ml TRIzol by using a motorized

homogenizer. Homogenates were incubated at room temperature for 5min. 0.2v/v of chloroform (100µl) was added and then the tube were briefly shaken to mix before incubation at room temperature for 3min. Samples were then centrifuged at 12,000 rpm for 15min at 4°C to separate RNA. The upper aqueous phase (containing RNA) was transferred to a fresh eppendorf tube. 1v/v of 70% ethanol was then added to the cleared lysate and mixed immediately. Up to 700µl of samples was applied to an RNeasy mini column (QIAGEN, Crawley-West Sussex) placed in a 2ml collection tube. The mini-column was centrifuged for 15s at 12,000 rpm and the flow-through was discarded. 700µl BW1 buffer (applied with the RNeasy mini column kit) was added in the column and it was centrifuged for 15s at 12,000 rpm. Flow-through was discarded. 500µl RPE buffer (applied with the RNeasy mini column kit) was added in each column and the column was spun, spin for 2mins at 12,000 rpm to wash the spin column membrane. The column was placed in a new 2ml collection tube and centrifuged at full speed for 1min. The RNeasy column was placed in a new 1.5ml collection tube. 30-50µl RNase-free water was directly added to the spin column membrane, incubated for 1min, and then centrifuged for 1min to elute. The RNA concentration was measured using a Nanodrop spectrophotometer (Thermo, Wilmington, USA)

2.13.3 Extraction of RNA from isolated islets (Matrix method)

Around 200 islets were added directly to 100µl Trizol. 20µl chloroform per 100µl Trizol was added to each sample and this was vortexed for 15 seconds and allowed to stand at room temperature for 2 to 3mins. Samples were transferred to ice for another 15mins and then centrifuged at 12000rpm at 4°C. The upper aqueous phase was transferred to a fresh eppendorf tube and 12µl Matrix (Rnaid matrix, Anachem, Texas, US) was then added and mixed to bind the RNA. To allow RNA binding to RNA matrix, samples were vortexed and gently agitated for 5mins and then centrifuged at 12,000g for 1min at 4°C to pellet the matrix. The supernatant was removed and the RNA pellet was then washed in 500µl RNA wash concentrate (Anachem, Luton, UK) diluted to contain 30% v/v absolute ethanol. The washed matrix was pelleted at 12,000g for 1min at 4°C. The washing step was repeated for three times. Following

washing the ethanol was discarded and the RNA pellets were allowed to air dry at room temperature for 5min. Pellets were resuspended in 12µl DEPC water and incubated in 55°C hotblock for 12mins to elute the RNA from the matrix. The RNA was separated from the matrix by centrigugation at 12000rpm for 2 minutes. RNA concentration was measured by Nanodrop spectrophotometer prior to reverse transcriptase reaction.

2.14 Reverse transcriptase reaction (RT)

Reverse transcriptase reactions were carried out using the Super Script III First-Strand Synthesis System to synthesize cDNA from total RNA. 1µl 10x DNase I buffer (Invitrogen) was added to 20µg RNA per sample in order to eliminate all genomic DNA contamination from RNA samples. Ultrapure water was used to adjust the final volume to 10µl and the samples were incubated at 22°C for 15mins. 1µl of EDTA was added to each tube and incubated at 65°C for another 15mins to inhibit DNase I. Samples were then kept on ice. Reverse transcription was carried out in a total volume of 20µl, containing 5µl RNA, 15µl cDNA synthesis mix consisting of 1µl Oligo dT primers (Promega) for specific hybridising to 3′ poly A tails in mRNA, 1µl dNTPs (Promega), 3µl 10x RT buffer (100mM Tris-HCl, 500mM KCl, 1% Triton-X100, Promega), 4µl 25mM MgCl (Promega), 2µl 0.1M DTT (Promega), 1µl RNase OUT, and 1µl SuperScript III (Promega). Samples were incubated at 50°C for 50mins. The reaction was terminated at 85°C for 5mins and then chilled on ice. Samples were stored at -20°C for further experiments (2.15).

2.15 PCR for 11β-HSD1 promoter expression

PCR reactions (50µl) contained 2µl RT reactions, 1.25U GoTaq (Promega), 30pmol of forward and reverse primer for each promoter or gene studied (promoter primers were kindly provided by Pr. Karen Chapman) (Bruley et al. 2006). GAPDH and 11β-HSD1 primers were used to control the quality of the RNA.

Primers were:

Promoter1 (P1): 5′- GGAGCCGCACTTATCTGAA -3′ (upstream exon1A, forward primer P1),

Promoter2 (P2): 5′- GGAGGTTGTAGAAAGCTCTG -3′ (upstream exon 1B, forward primer P2),

Promoter3 (P3): 5′- GTATGGAAAGCAAGACAAGG -3′ (upstream an intron before exon 3, forward primer P3)

P868: 5′-AGGATCCAGAGCAAACTTGCTTGCA-3′ (reverse primer for P1, P2, P3),

$P_{HSD:}$ 5′-AAAGCTTGTCACTGGGGCCAGCAAA-3′ (forward primer for HSD1),

P869: 5′- AGGATCCAGAGCAAACTTGCTTGCA-3′ (reverse primer for HSD1)

$P_{GAP:}$ 5′- GCCAAGGTCATCCATGACAAC-3′ (forward primer for GAPDH)

$P_{GAP:}$ 5′- AGTGTAGCCCAAGATGCCCTT-3′ (reverse primer for GAPDH)

The PCR program was 95°C for 45secs, then 40 cycles of 90°C for 45secs, 60°C for 30secs, 72°C for 90secs, then finally 72°C for 5mins and 4°C for 10mins. Samples were run on a 2% agarose gel in 0.5x TBE buffer and compared to negative and positive controls and a 100bp DNA ladder (Invitrogen).

2.16 Immunohistochemistry (IHC)

Pancreata were fixed in 10% formalin (sigma) for 24 hrs and transferred to 70% ethanol, and automatically dehydrated in a tissue processor TP1050 (Leica, Ramsey, USA) that embedded tissue fragments in paraffin by Histocentre 3 (Thermo Shandon). Tissue sections were cut by microtome. Tissue sections were deparaffinized and rehydrated by incubating twice in xylene for 5mins, then placed in sequential washes of 100% (2x), 95%, and 80% ethanol solutions for 20 seconds each with a final wash in water. Sections were then put in a pressure cooker and boiled in 10mM citrate buffer (pH 6.0) for 5mins for antigen retrieval (breaking the protein cross-links formed by formalin), and cooled at room temperature for another 20mins. Sections were incubated for 30mins in 3% hydrogen peroxide in methanol which is commonly used to block endogenous peroxidase activity. Sections were then blocked by blocking buffer (20% goat serum with 5% BSA, in TBS (table 2-3))

for 30mins, followed by Avidin and Biotin (Vector Lab, Peterborough, UK) blocking for 15mins each, and between each block sections were washed twice with washing buffer (TBS, pH 7.4). Sections were incubated with primary antibodies (Table 2-1, described in chapter 5) at 4°C overnight. Antibodies were diluted in blocking buffer. Slides were washed twice in washing buffer, and then incubated with secondary antibodies diluted in blocking buffer. Secondary anti-bodies were biotinylated anti-guinea pig (1:400, Abcam) or biotinylated goat anti-rabbit (1:500, Dako, Cambridge, UK) and incubation was at room temperature for 30mins, followed by two washing cycles. Slides were incubated with Vectastain ELITE ABC-Peroxidase Kit (Vector lab) for 30mins, rinsed with washing buffer as before. Signal was developed in freshly prepared diaminobenzidine (DAB) solution (DAB Peroxidase Substrate Kit, Vector lab) following the manufacturer's instructions. The reaction was stopped by washing in TBS until a uniform brown color became visible on the sections. Sections were counter-stained with Haematoxylin Solution (Sigma), rinsed under running water, decolorized with 1% acid alcohol for 2 seconds, washed with running water, blue-stained by Scotts tap water, washed under running water and then rehydrated, followed by incubating in 70%, 80%, 95%, 100% (2x) ethanol, and finally xylene for 5mins. Slides were mounted with a cover glass (VWR) in an aqueous medium.

All negative controls were used the appropriate same original serum as the staining first antibody at the same concentration.

2.17 Immunofluorescence (IF)

Sections were fixed in paraffin and processed with the following steps. Sections were deparaffinized, rehydrated, subjected to antigen retrieval, inactivated by peroxidase and then blocked by blocking buffer. Sections were then incubated with primary (table 2-3) rabbit anti-ki67 (1:3000) antibody at 4°C overnight. Slides were washed twice with washing buffer (TBS, PH 7.4), and then incubated with goat anti-rabbit peroxidase (Abcam) at room temperature for 30mins, followed by 2 further washes. Slides were incubated with Tyramide green 488 (Tyramide signal amplification kit, Cambridge, UK) to amplify the signal at room temperature for 10mins, followed by

two washes, and then slides were stored in the dark. 10mM citrate buffer was boiled by microwave for 4mins, and then the slides were added to the boiled citrate buffer for another 2.5mins, cooled for 30mins, and then washed with washing buffer for 5mins. The slides were blocked for 30mins and then incubated with a second primary antibody rabbit anti-PDX-1 (1:1000) antibody at 4°C overnight. On the third day, slides were washed twice, incubated with goat anti-rabbit Alexa Fluor 546 (Molecular probes, Paisley, UK) at a 1:200 dilution in PBS at room temperature for 1 hour. Slides were washed twice. Dapi (Sigma, diluted 1:1000 in PBS) was used to stain nuclei by incubating sections for 10mins. The slides were mounted with PermaFluor (Thermo) and visualised using a Leica fluorescence microscope.

2.18 Quantification of IHC/IF

Quantification of Foxp3 was carried out by using computerized image analysis. Three sets of paraffin sections from each pancreas were made for Foxp3 and each section was cut at intervals of 100μm. The number of Foxp3 positive cells was determined from light microscopic image and counted using Zeiss (KS300, 3.0) software throughout the section. Pancreas section area was measured by MCID Basic 7.0 software.

Quantification for PDX1 and Ki67 was performed using Image J software on light microscopic images. PDX1 and Ki67 positive cells were measured within the islets as defined morphologically by the islet capsule boundary. PDX1 and Ki67 double-positive cells were counted on each section. The rate of proliferating β-cells was presented as the ratio of PDX1 and Ki67 double-positive cells to PDX1-positive cells in each islet and this was then averaged for each mouse within each group.

Table 2-1. List of primary antibodies for IHC and IF

IHC	Anti-Insulin	Guinea pig anti-mouse (Abcam, Cambridge, UK)	1:300
IHC	Anti-PDX1	Rabbit anti-mouse (Chemicon international inc)	1:6000
IHC	Anti-Mac-2	Rabbit anti-mouse (Cedarline, ON, Canada)	1:150
IHC	Anti-Foxp3	Rat anti-mouse (eBioscence, Hatfield, UK)	1:150
IHC	Anti-sox9	Rabbit anti-mouse (Chemicon)	1:8000
IHC	Anti-Neurogenin 3	Rabbit anti-mouse (Chemicon)	1:1000
IHC	Anti-Caspase3	Rabbit anti-mouse (Cell signaling, Hertfordshire, UK)	1:100
IF	Anti-PDX1	Rabbit anti-mouse (Chemicon international inc)	1:1000
IF	Anti-ki67	Rabbit anti-mouse (DAKO)	1:3000

2.19 TUNEL assay for apoptosis in pancreatic sections

Slides were incubated at 60°C for 15mins, dewaxed, rehydrated and subjected to antigen retrieval as described in 2.2.10. After washing with PBS for 3 times, five minutes each wash, slides were fixed with 4% (v/v) formaldehyde in PBS for 15 min at room temperature and then washed twice with PBS. 100µl of Proteinase K (20µg/ml) was added to each slide in a humidity chamber, ensuring the tissue section was fully covered. Incubation was for 10min at room temperature and was stopped by washing in PBS once. Slides were then fixed with 4% (v/v) formaldehyde in PBS for another 5 min at room temperature, and then washed with PBS. The DeadEnd™ Fluorometric TUNEL assay kit (Promega) was used. 100µl of the equilibration buffer was added to each slide in a humidity chamber and incubated at room temperature for 10min. During this time, reaction mixtures were prepared. 5µl of nucleotide mix and 1µl of rTdT enzyme was mixed with 45µl equilibration buffer for each slide, and a negative control was prepared by mixing 5µl nucleotide mix and 46µl equilibration buffer. Equilibration buffer was drained off and 50µl of the reaction mixture was added to each slide and covered with a plastic coverslip. Slides were incubated in a humid dark chamber for 1 hour at 37°C. Slides were kept in the dark. After reacting, coverslips were removed and the slides were incubated in 2× SSC for 15mins followed by washing in PBS for 5min. Dapi was used to stain nuclei. The slides were mounted with PermaFluor using and images visualised using Leica fluorescence microscope (2.18).

2.20 Western Blot

0.5ml lysis buffer (table 2-3) was added to each pancreas and they were homogenized using a motorized homogenizer (as above 2.11). The homogenates were centrifuged at 12000rpm for 15mins at 4°C. The aqueous solution was transferred to new tubes. A protein assay was used to measure protein concentration (2.12). Equal amounts of protein were mixed with 2µl of 6x loading buffer (table 2-3) and water was used to adjust the final volume to 10µl. Samples were heated at 100°C

for 5mins, cooled on ice, quickly spun and then kept on ice. Samples and 10µl molecular weight marker (See Blue, Invitrogen) were loaded onto SDS-PAGE gels (table 2-3) and run at 100 Volts for 2hours to separate proteins. Proteins were electrotransfered from the gel to a PVDF membrane (GE Healthcare) in transfer buffer (1x, table 2-3) at 200mA for 2 hours. The transfer system was kept cool during electrotransfer with an inset ice-block (previously chilled to -20°C). After transfer, the membrane was washed in TBS-T (table 2-3) 0.1% for 5mins and then incubated in TBS-T with 5% milk for 1 hours at room temperature to block. The membrane was rinsed 3 times for 5mins each with TBST-T and incubated with a rabbit primary antibody (anti-cleaved caspase-3 at 1:1000, Cell Signaling) in TBS-T 5% BSA overnight at 4°C. The membrane was washed three times for 5mins with TBS-T, then incubated with an anti-rabbit HRP-conjugated secondary antibody (1:2000, cell signaling) in TBST-T with 5% milk with gentle agitation for 2 hours at room temperature. The membrane was washed 3 times for 5mins each with TBS-T. The membrane was incubated with 1ml developing solution (Supersignal West Pico kit, Pierce, Thermo) for 1 minute. Excess developing solution was drained; the membrane was wrapped in saran wrap and then exposed to X-Ray film for 3mins. Signal was developed by exposing the film through a Konica X-Ray developer (Konica, FL, US).

2.21 Data analysis and statistics

Streptozotocin experiments, plasma assays, IHC and IF were analysed with an initial blinding step with subsequent code-breaking after quantification. Data are presented as the mean ± S.E.M. Statistical analysis was performed by Graph Pad (GraphPad Prism 4 Demo). The groups were compared using either Student's t-test (for a simple comparison of two groups) or one-way ANOVA (Tukey comparison all groups). Two-way ANOVA (Holm-Sidak, pairwise multiple comparison) was used to compare glucose and treatment interactions. A P value of <0.05 was considered to show a statistically significant difference between groups.

Table 2-2. List of equipment

TECHNE TC-512 PCR machine	Techne Inc.
UV transilluminator	Uvitec Ltd
Hybridization oven	Weiss-Gallenkamp
Optical light microscope	Zeiss
Spectrophotometer	Molecular Device
Radioimaging detector ,FLA-2000	Fujifilm
Gene Quant RNA/DNA calculator	Pharmacia
Motorized homogenizer	IKA
Fluorescence microscope	Leica
Konica SRX-101A X-Ray developer	Konica
LightCycler 480 thermocycler	Roche
Nanodrop spectrophotometer	Thermo

Table 2-3. List of buffers and solutions

Tail Buffer	0.5ml 1M Tris, 4ml 250mM EDTA, 0.2ml 5M NaCl, 1ml 10% SDS, 4.3ml dH_2O
TE Buffer	606mg Tris, 18.6mg EDTA, make up to 500ml
6M ammonium acetate	4.62g ammonium acetate was dissolved in 10ml dH_2O
10x TBE buffer	108g Tris base, 55g Boric acid, 40ml 0.5M EDTA were dissolved in 1L dH_2O
50 x TAE buffer	121g Tris, 28.5ml glacial acetic acid and 50ml 0.5M, EDTA (pH8.0) were dissolved in 500ml dH2O
DNA loading buffer	39ml 2M NaH_2PO_4, 61ml 2M Na_2HPO_4, Glycerol 1v/v, Bromophenol Blue
0.15M HCL	6.48ml HCL was diluted in 500ml dH_2O
0.5M NaOH, 1.5M NaCl	20g NaOH, 87g NaCl, were dissolved in 1L dH_2O
1M Tris, 1.5M NaCl, 1mM EDTA	121g Tris, 87g NaCl, 0.372g EDTA, were dissolved in 1L dH_2O, pH 7.2
20x SSC	175.3g NaCl, 88.22g Citrate Na, were dissolved in 1L dH_2O.
10% SDS	10g SDS was added into 80ml dH_2O and heated to 60°C until completely dissolved. pH was adjusted to 7.5 and the volume was up to 100ml with dH_2O
PBS buffer (10x)	80g NaCl, 29g Na_2HPO_4, 2g KH_2PO_4, 2g KCl, were dissolved in 1L dH_2O, pH 7.4
0.1MCitrate buffer	42.02g citric acid, made up to 2L, pH6.0

Borate buffer	8.25g boric acid, 2.7g NaOH, 5g BSA, make up to 1L, pH 7.4
KREBS Ringer Phosphate buffer	6.9g NaCl, 373mg KCl, 296mg $MgSO_4$, 800ml dH_2O; 120ml Na_2HPO_4 (7.1g Na_2HPO_4 dissolved in 500ml dH_2O), 3ml NaH_2PO_4 (6g NaH_2PO_4 dissolved in 500ml dH_2O) were mixed into the above solution, pH 7.4; 2.28ml was taken from freshly prepared 6.1% $CaCl_2$ (0.61g $CaCl_2$ dissolved in 10ml dH_2O); Made up to 1L dH_2O, stored at 4°C, 0.1% BSA was added before use.
0.1M citrate acid	1.92g Citric acid was dissolved in 100ml dH_2O,
0.1M Nacitrate dihydrate	14.7g Nacitrate dehydrate was dissolved in 500ml dH_2O
10mM citrate buffer	5ml 0.1M citrate acid, 5ml 0.1M Nacitrate dehydrate were dissolved in 100ml dH_2O, pH4.5
0.5M Tris-HCl	121.14g Tris methylamine, made up to 2L, pH 7.4
50mM TBS (Tris buffered saline)	100ml 0.5M Tris-HCl, 8.5g NaCl, made up to 1L
Lysis buffer	50mmol/l Tris, pH 7.4, 0.27 mol/l sucrose, 1mmol/l sodium orthovanadate, pH 10, 1mmol/l EDTA, 1mmol/l EGTA, 10mmol/l sodium β-glycerophosphate, 50mmol/l NaF, 5mmol/l sodium pyrophosphate, 1%[wt/vol] Triton X-100, 0.1% [vol/vol] 2-mercaptoethanol, and protease inhibitors—EDTA free tablets (Roche)

SDS-PAGE gel	12% separating gel: 3.35ml dH_2O, 2.5ml 1.5M Tris (PH 8.8), 0.1ml 10%SDS, 4ml 30%Acrylamide, 0.75ml 10%APS, 0.01ml Temed; Stacking gel: 2.85ml dH_2O, 1.25ml 0.5M Tris (PH 6.8), 0.05ml 10%SDS, 0.85ml 30%Acrylamide, 0.025ml 10%APS, 0.005ml Temed.
Running buffer (10x)	30g Tris, 144g Glycine, 10g SDS, made to 1L dH_2O.
Loading buffer (6x)	1.8ml 2M Tris (PH 6.8), 3ml Glycerol, 0.3ml 0.5% Bromophenol Blue, 1.8g SDS, 925mg DTT, made up to 10ml with dH_2O.
Transfer buffer (10x)	24.2g Tris, 112.3g Glycine made to 1L dH_2O.
Transfer buffer (1x)	100ml transfer buffer (10x), 800ml dH_2O, 100ml Methanol
TBS-T 0.1%	1L TBS, 1ml Tween-20

Chapter 3

The Role and Regulation of 11β-HSD1 in Pancreatic Islets

3 The role and regulation of 11β-HSD1 in pancreatic islets

3.1 Introduction

Glucocorticoids play a critical role in regulating glucose homeostasis. The mechanisms of their effects involve in increasing hepatic gluconeogenesis and glucose output (Barthel and Schmoll 2003) and diminishing insulin sensitivity in muscle (Dimitriadis et al. 1997; Smith and Muscat 2005) and adipose tissue (Sakoda et al. 2000).

GC access to its nuclear receptor GR is tightly regulated by the intracellular enzyme 11β-HSD1 which catalyses the local reactivation of GCs within tissues when H6PDH is abundant. This enzyme is expressed in both rodent and human pancreatic islets, and it is highly expressed in β-cells (Turban et al. unpublished), although it has been found expressed in α-cells (Swali et al. 2008). Little is known about the regulation of 11β-HSD1 in pancreatic islets, although 11β-HSD1 activity in isolated islets is increased by 11DHC *in vitro* (Davani et al. 2000; Ortsater et al. 2005). As described in chapter 1.5.2 there are three promoters which regulate 11β-HSD1 transcription. P1 predominates in lung, P2 predominates in liver, adipose tissue, and brain and P3 is utilized in the kidney and is believed to encode a non-enzymatically active 11β-HSD1 protein. However, the predominant promoter used to drive 11β-HSD1 gene expression in pancreatic islets is unknown. Although the regulation of P1 and P3 promoters has not been yet characterized, the C/EBP families are the major transcription factors regulating the 11β-HSD1 P2 promoter (McKnight et al. 1989; Darlington et al. 1995; Croniger et al. 1998; Poli 1998). The proinflammatory IL-1 and TNF-α cytokines increase 11β-HSD1 mRNA levels in glomerular mesangial cells (GMC) (Escher et al. 1997), granulosa cells (Tetsuka et al. 1999), human aoric smooth muscle cells (Cai et al. 2001), human bronchial smooth muscle cells (Cai et al. 2001), osteoblast cells (Zulewski et al. 2001), human ovarian surface

epithelial cells (Yong et al. 2002), and human preadipocytes (Tomlinson et al. 2001). Furthermore, TNFα and IL-1 increase 11β-HSD1 expression by inducing the binding of C/EBPβ to the P2 promoter in hepatoma (HepG2) cells (Ignatova et al. 2009) and human fetal lung fibroblasts (Yang et al. 2009). The regulation of 11β-HSD1 by insulin and GCs has been extensively studied. However, the literature is inconsistent and incomplete suggesting differential effects that are highly tissue specific and duration dependent in operation. In general, GCs act as a stimulator of 11β-HSD1, while insulin is an inhibitor of the enzyme expression and/or activity in skin fibroblasts (Hammami and Siiteri 1991), testis (Nwe et al. 2000), preadipocytes (Kim et al. 2007), and hepatocytes (Liu et al. 1996; Voice et al. 1996). GCs dose -ependently increase 11β-HSD1 expression in human skeletal muscle (Whorwood et al. 2002), and temporally regulate 11β-HSD1 expression and activity in rat liver and hippocampus *in vivo* (Moisan et al. 1990; Jamieson et al. 2000). In adipocytes, GCs down-regulate 11β-HSD1 *in vitro* (Napolitano et al. 1998; Balachandran et al. 2008) but up-regulate 11β-HSD1 *in vivo* (Livingstone et al. 2000; Balachandran et al. 2008). Insulin has also stimulates 11β-HSD1 expression and activity in adipocytes both *in vivo* and *in vitro* (Wake et al. 2006; Westerbacka et al. 2006; Balachandran et al. 2008).

Previous studies report 11β-HSD1 mRNA and activity were elevated in islets of obese and diabetic rodent models such as ob/ob mice (Davani et al. 2000; Ortsater et al. 2005) and ZDF rats (Duplomb et al. 2004). This suggested altered glucose metabolism itself may regulate the expression of 11β-HSD1. Nevertheless, these studies failed to find a direct regulatory effect of glucose, leptin, or a convincing effect of free fatty acids on islet 11β-HSD1 (Duplomb et al. 2004). Only the treatment with a PPARγ agonist thiazolidinedione (troglitazone), which also inhibits the expression of 11β-HSD1 in preadipocytes (Berger et al. 2001), showed a small, direct effect to suppress 11β-HSD1 in ZDF islets (Duplomb et al. 2004). Therefore 11β-HSD1 regulation by cytokines and hormones remains unclear and it is of key importance to address this question to fully understand the physiological role of this enzyme in islet function. The present work was undertaken to obtain a better

understanding of the role and regulation of 11β-HSD1 activity, in pancreatic islets, in particular in response to known diabetogenic factors.

3.2 Aims

1. To determine the predominant enzyme directionalty of 11β-HSD1 in pancreatic islets;
2. To identify which promoter of 11β-HSD1 is used in pancreatic islets;
3. To investigate which factors regulate 11β-HSD1 activity in pancreatic islets.

3.3 Experimental design

3.3.1 11β-HSD1 activity and directionality in pancreatic islets

11β-HSD1 possesses both reductase and dehydrogenase activities in its purified form and in tissue homogenates. The reductase is the predominant function in most intact cells and *in vivo* (Low et al. 1994; Jamieson et al. 1995) when the predominant co-factor providing enzyme H6PDH is also expressed. To find out which activity of 11β-HSD1 is predominant in pancreatic islets, batches of 100 islets were isolated (Described in 2.7), and incubated with [^{3}H] labeled 11-dehydrocorticosterone (A compound) to measure reductase activity; while [^{3}H] labeled corticosterone (B compound) was incubated to test the dehydrogenase activity. Furthermore, to determine the potential effect of glucose metabolism on islet 11β-HSD1 reductase and dehydrogenase activities, islets were incubated in either low glucose concentration (2.8mM) or high glucose concentration (16.8Mm). Islets were incubated for 24 hours and medium was extracted to measure the conversion of A to B, or B to A by TLC methods (Described in 2.9).

3.3.2 Determining the 11β-HSD1 promoter use (P1, P2 or P3) in pancreas and isolated islets

For a better understanding of the regulation of 11β-HSD1 in pancreas and islets, it was important to determine which promoters are employed to regulate 11β-HSD1 expression in pancreas and islets. There are three known promoters that have been found to regulate 11β-HSD1 transcription (Described in 1.5.2). Although the regulators for P1 and P3 are still largely unknown, the P2 is regulated by C/EBP transcription factors (Described in 1.5.3). RNA was extracted from whole mouse pancreas or isolated islets. Reverse transcription followed by PCR with specific designed primers for the three different promoters (a gift from Dr. Karen Chapman).

3.3.3 The effects of glucose, fatty acids, insulin, dexamethasone and cytokines on islet 11β-HSD1 reductase activity *in vitro*

Obesity and T2D are characterized by high plasma glucose, fatty acids, insulin, and certain cytokines (Boden and Shulman 2002). Glucocorticoid action is also elevated, at least within certain tissues (Morton and Seckl 2008). These diabetes-associated factors might be directly responsible for excessively increased islet 11β-HSD1 activity found in some diabetic rodents (Davani et al. 2000; Livingstone et al. 2000; Masuzaki et al. 2001). To investigate this, islet 11β-HSD1 activity was measured by incubating pancreatic islets with different factors.

In this case, a physiological glucose concentration (5mM) and a stimulatory (with regards to insulin secretion, 20mM) were used to assess glucose effects on islet 11β-HSD1 activity. To control for potential confounding osmotic effects of the higher glucose concentration, sorbitol, a glucose "analogue" without a key hydroxyl group was used at the same concentration (20mM). 11β-HSD1 activity was measured from the cultured medium by TLC.

Fatty acids potentiate glucose-stimulated insulin secretion from normal pancreatic β-cells and also play a role in the pathogenesis of β-cell dysfunction when they accumulate (lipotoxicity) within the islets in T2D (McGarry and Dobbins 1999; Poitout and Robertson 2002). Palmitate which is an abundant dietary-derived fatty acid in human plasma, was used as a reference compound to study the influence of fatty acids on islet 11β-HSD1 activity *in vitro* (Warnotte et al. 1999). Islets were isolated from the mice, and cultured in the absence or presence of 0.5mM palmitate (high physiological level in obese, diabetic range) (Jacqueminet et al. 2000) along with basal (5mM) or high (20mM) glucose concentrations to mimic the combination found in the type 2 diabetic condition.

Plasma TNFα is increased with morbid obesity (Hotamisligil et al. 1993) and stimulates 11β-HSD1 activity in many cell lines (details in chapter 1.5.3). Its

regulatory effect was mediated by C/EBPβ on the P2 promoter of 11β-HSD1 in HepG2 cells (Ignatova et al. 2009). To evaluate TNFα effect on islets 11β-HSD1 activity, 1ng/ml TNFα was used, a concentration that stimulated 11β-HSD1 activity in human adipose stromal cells (Handoko et al. 2000).

Although plasma glucocorticoids are largely normal during obesity and the early stage of diabetes, they are elevated in the later stages of diabetes, at least in rodents (Shimomura et al. 1987). Glucocorticoids regulate 11β-HSD1 activity in several tissues and cells apart from islets (Tomlinson et al. 2004). Dexamethasone is a synthetic glucocorticoid widely used as an anti-inflammatory drug (reviewed in (Swartz and Dluhy 1978; Holland and Taylor 1991; McEwen et al. 1997)). The concentration of dexamethasone chosen to test effects on islets 11β-HSD1 activity was 100nM, comparable to that used for investigating 11β-HSD1 expression in adipocytes (Napolitano et al. 1998).

Circulating insulin is elevated secondary to insulin resistance in T2D. In insulin resistant states, insulin levels can reach up to 10nM or greater (Hollenbeck et al. 1984). The effects of insulin on regulating 11β-HSD1 expression and activity is not clear and seems to depend on the tissue or cell types studied (Moisan et al. 1990; Hammami and Siiteri 1991; Liu et al. 1996; Napolitano et al. 1998; Jamieson et al. 1999; Handoko et al. 2000; Nwe et al. 2000; Koistinen et al. 2004; Sandeep et al. 2005; Wake et al. 2006; Kim et al. 2007; Balachandran et al. 2008). 10nM insulin was chosen to reflect a supraphysiological level found in compensatory hyperinsulinaemia of insulin resistance.

3.4 Results

3.4.1 11β-HSD1 is predominantly a reductase in pancreatic islets

The reductase activity of 11β-HSD1 was significantly higher (ten-fold) than the dehydrogenase in pancreatic islets. 16.8mM glucose had no significant effect on either reductase or dehydrogenase activities of 11β-HSD1 in islets in this studies, and the directionality was unaffected overall (Figure 3-1).

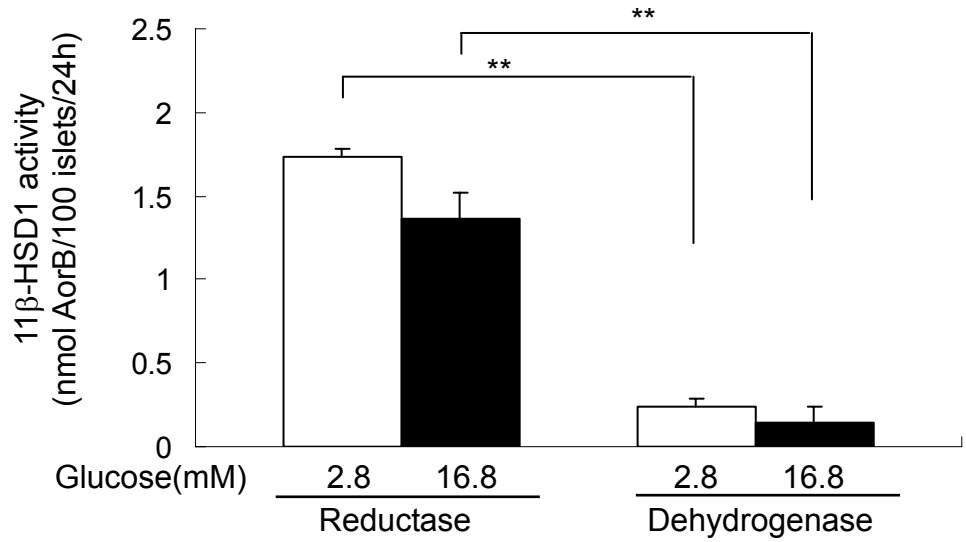


Figure 3-1. 11β-HSD1 activity is predominantly reductase in pancreatic islets. 11β-HSD1 activities were measured by TLC. The reductase activity is represented by conversion of A to B, while dehydrogenase by the conversion of B to A. Reductase is significantly higher than dehydrogenase of 11β-HSD1 in islets in both low glucose concentration (A) and high glucose concentration (B). The islets were isolated from C57BL/KsJ (KsJ) mice. Values represented the mean ± S.E.M, **P<0.01 (One-way ANOVA, Tukey's Multiple Comparison Test), reductase group n=9, dehydrogenase group n=4.

3.4.2 The P1 and P2 11β-HSD1 promoters are predominantly used for 11β-HSD1 expression in pancreas and islets

Pancreas and pancreatic islets were isolated from mice and RNA was extracted. Reverse transcription followed by PCR with specific primers for the three different promoters were run to find out which promoters are predominant in whole pancreas and islets. For islets and pancreas the PCR produced a strong band at the expected size of 600bp using primers specific for P1 (encoding exon 1A) and P2 (encoding exon1 B), indicating the robust expression of promoters 1 and 2 in pancreas and islets (Figure 3-2). Both GAPDH and 11β-HSD1 controls were positive indicating the RNA was of good quality.

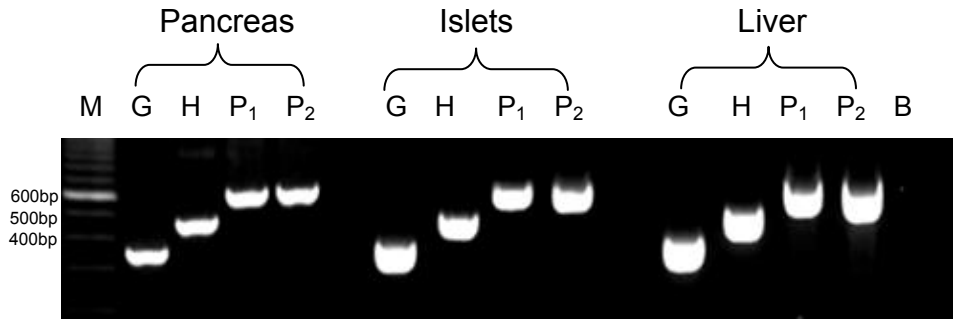


Figure 3-2. P1 and P2 are the predominant promoters in pancreas and islets. PCR gel visualized by UV trans illuminator showing P1 and P2 specific bands at 600bp from pancreas and islets RNA. Liver was used as a positive control showing P1 and P2; negative controls (B) contain no RNA (H_2O); GAPDH (G) and 11β-HSD1 (H) primers were used as a control for the RNA quality.100bp marker (M).

3.4.3 The effects of nutrients, hormones and cytokines on pancreatic islet 11β-HSD1 activities *in vitro*

To address the effects of glucose on 11β-HSD1 activity, islets were isolated from KsJ mice, and cultured with basal (5mM) glucose level, or a maximal stimulatory high glucose concentration (20mM) for 24 hours. 20mM sorbitol was used as an osmotic control. Compared to low glucose, there was a significant decrease of 11β-HSD1 activity with high glucose. 11β-HSD1 activity was not affected by 20mM sorbitol compared to low glucose concentration, but was higher than 20mM glucose (Figure 3-3).

To investigate the effect of free fatty acid on 11β-HSD1 activity, 0.5mM palmitate was used. 0.5mM palmitate significantly increased islet 11β-HSD1 activity with low glucose incubation and reversed high glucose-mediated inhibitory effects on islet 11β-HSD1 activity (Figure 3-3).

To determine the effect of TNFα on 11β-HSD1 activity, 1ng/ml TNFα was used. TNFα (1ng/ml) had no significant effect on 11β-HSD1 activity in the presence of low glucose. However, TNFα clearly prevents high glucose-induced inhibition of 11β-HSD1 activity (Figure 3-3).

To evaluate the effect of active GCs on 11β-HSD1 activity, 100nM dexamethasone was used. 100nM dexamethasone had no effect on islet 11β-HSD1 activity either with low or high glucose (Figure 3-3)

10nM insulin clearly inhibited islet 11β-HSD1 activity in the presence of low glucose condition but had no effect on high glucose-mediated inhibition (Figure 3-3).

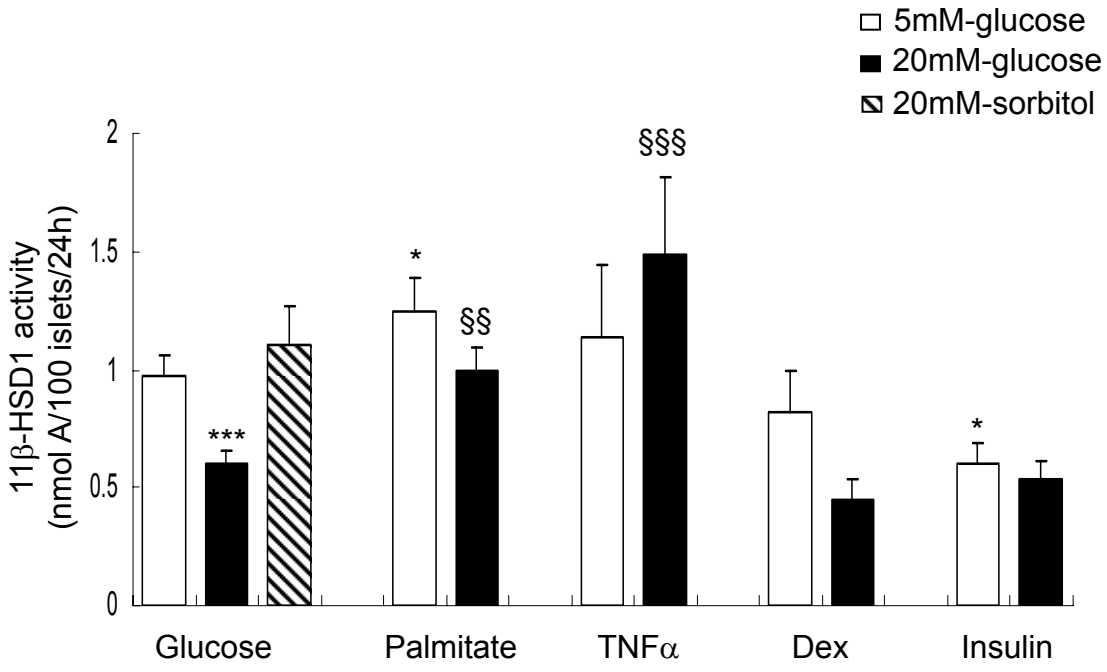


Figure 3-3. Effects of glucose, palmitate, TNFα, dexamethasone and insulin on islet 11β-HSD1 reductase activity. Islets were isolated from KsJ mice, incubated in basal glucose condition (5mM Glucose, blank bars) or high glucose level (20mM Glucose, solid bars) with 20mM Sorbitol (strip bars), 0.5mM palmitate, 1ng/ml TNFα, 10nM insulin, and 100nM dexamethasone. Values represent the mean ± S.E.M, *** P<0.0001, *P<0.05 vs. 5mM glucose without treatment, Two-way ANOVA, n=6, §§§ P<0.0001, §§ P<0.01, vs. 20mM glucose without any treatment, Two-way ANOVA, Holm-Sidak, pairwise multiple comparison, n=6.

3.5 Discussion

Glucocorticoids have profound effects on glucose metabolism under both normal and pathologic conditions. Considering the free unbounded inactive glucocorticoids (11DHC in rodents) are predominant in the circulation (Harris et al. 2001), they can be locally activated by the action of 11β-HSD1 in tissues. There is growing evidence that 11β-HSD1 is a bidirectional enzyme, in some cells (Lakshmi and Monder 1988; Agarwal et al. 1989; Bujalska et al. 1997; Atanasov et al. 2004; Lavery et al. 2006; Dzyakanchuk et al. 2009). Therefore, demonstrating which activity of this enzyme predominates in islets is important. 11β-HSD1 is expressed in pancreatic islets of KsJ mice as previously reported in C57BL/6J, $Lep^{ob/ob}$ mice and human islets (Davani et al. 2000; Ortsater et al. 2005; Swali et al. 2008). Islet 11β-HSD1 activity is predominantly reductase. We found the reductase activity of 11β-HSD1 was ten fold higher than dehydrogenase activity in isolated islets of KsJ wild type mice *in vitro*. Furthermore, increasing glucose in the culture medium did not affect the directionality of this enzyme, suggesting the reductase activity is the predominant activity of 11β-HSD1 in pancreatic islets and this is not changed in obesity or diabetic conditions.

11β-HSD1 activity is higher in some obesity induced diabetes animal models, such as $Lep^{ob/ob}$ mice (Ortsater et al. 2005) and ZDF rats (Duplomb et al. 2004). Thus, the regulation of 11β-HSD1 in pancreatic islets is important to understand. RT-PCR analysis revealed that P1 and P2 were both involved in regulating 11β-HSD1 transcription of the isolated islets from KsJ mice as in liver or adipose tissue (Bruley et al. 2006). This suggests that 11β-HSD1 expression might be regulated in a similar way as shown in liver and adipose tissues and comparably by inflammatory factors, cytokines and nutrients (Liu et al. 1996; Voice et al. 1996; Napolitano et al. 1998; Jamieson et al. 2000; Livingstone et al. 2000; Tomlinson et al. 2004; Kim et al. 2007; Balachandran et al. 2008). However, whilst this work shows that both promoters are used, the relative level of each promoter remains to be quantified.

We have attempted to look at the effect of several diabetogenic factors on islet 11β-HSD1 activity. There is a significant inhibitory effect of high glucose on 11β-HSD1 activity in isolated islets. The lack of effect of sorbitol, a glucose "analogue", indicates the specific effect of glucose or one of its metabolites and not an osmolarity effect. This suggests that hyperglycaemia is not the cause of elevated 11β-HSD1 activity in islets (Duplomb et al. 2004; Ortsater et al. 2005). This also contrasts with the stimulatory effect of high glucose on 11β-HSD1 reductase activity in HEK-293 cell, hepatoma cells and unaltered 11β-HSD1 activity in 3T3-L1 adipocytes *in vitro* (Dzyakanchuk et al. 2009). Although this may be explained by different species and glucose levels used, it is more likely that high glucose regulates 11β-HSD1 activity differentially in distinct tissues and suggests a unique effect of glucose metabolism in this specialised glucose-response cell type (islets). A reduction in 11β-HSD1 activity is observed in high fat diet induced β-cell failure in KsJ mice (see 4.5.9). This is also in accordance with the fact that the presence of a high glucose concentration in the islet environment induces glucotoxicity by increasing ROS formation that suppresses insulin production (Robertson and Harmon 2006) which might interfere with the ER lumen environment where 11β-HSD1 is localised (Turban et al. unpublished).

Although three days in the presence of fatty acids (oleate/palmitate) failed to modify 11β-HSD1 mRNA level in isolated islets of pre-diabetic ZDF rats (Duplomb et al. 2004), increased saturated fatty acids (palmitate) elevated the 11β-HSD1 activity within 24hr in isolated islets from KsJ mice. This suggests that lipids have the ability to increase 11β-HSD1 activity and this might account for the increased level of activity seen in islets of obese mouse models (Davani et al. 2000; Ortsater et al. 2005) where high levels of FFA are present in the systemic circulation (Ingalls et al. 1950). Interestingly, high free fatty acid levels reverse the glucose-mediated suppression of islet 11β-HSD1 activity, suggesting lipid mediated regulation is dominant. It is currently unclear why KsJ islets show a lower 11β-HSD1 level on high fat diet, however, it is possible their increased β-cell failure susceptibility alters their

response to glucotoxicity and lipotoxicity.

TNFα is elevated in morbid obesity and stimulates 11β-HSD1 transcription through P2 in many cell lines (details in 1.5.3). No such work has been carried out on pancreatic islets. We show that TNFα reverses the inhibitory effects caused by high glucose on islet 11β-HSD1 activity. TNFα alone had no effect at physiological glucose concentration. This suggests the TNFα might contribute to the elevation of islet 11β-HSD1 activity in obesity and diabetes and is consistant with the increased inflammatory state of obesity and diabetes (Kolb and Mandrup-Poulsen 2005; Wellen and Hotamisligil 2005). Moreover, the increased 11β-HSD1 activity in islets by TNFα could activate GCs which may locally exert immunomodulatory effects (see 5.5.1.3).

Dexamethasone had no effects on islet 11β-HSD1 activity in both physiological and high glucose conditions. This lack of effect is surprisingly different from findings that GCs increase 11β-HSD1 activity in other tissue and cell types such as skin fibroblasts (Hammami and Siiteri 1991), testis (Nwe et al. 2000), preadipocytes (Kim et al. 2007), hepatocytes (Liu et al. 1996; Voice et al. 1996), human skeletal muscle (Whorwood et al. 2002), and adipocytes *in vivo* (Livingstone et al. 2000; Balachandran et al. 2008). Further in adipocytes *in vitro* (Napolitano et al. 1998; Balachandran et al. 2008) and rat liver *in vivo* (Nwe et al. 2000), GCs decrease the 11β-HSD1 activity. This important observation suggests that increased local generation of GC does not initiate a feed forward GC regulation of 11β-HSD1, and that elevated circulating GC (e.g. Cushing syndrome) may not alter islet 11β-HSD1 directly. Indeed, this finding implicates other factors as being responsible for altered 11β-HSD1 levels in islets in obesity and diabetes.

Insulin showed a significant inhibitory effect on 11β-HSD1 activity only at low glucose concentration. This is in agreement with the negative regulatory effects of insulin found in many other tissues and cell types (Moisan et al. 1990; Hammami and Siiteri 1991; Liu et al. 1996; Jamieson et al. 1999; Nwe et al. 2000; Sandeep et al.

2005; Kim et al. 2007). This suggests that high physiological insulin levels suppress islet 11β-HSD1, and that insulin resistance in the β-cell may therefore contribute to the elevated islet 11β-HSD1 found in obese and diabetic animals. That insulin mediated islet 11β-HSD1 suppression is abolished by high glucose suggests a physiological effect such that glucose metabolism effects dominate over basal state effects of insulin (e.g. during a meal when glucose and insulin are both transiently elevated).

In summary, we find the reductase is the predominant activity of 11β-HSD1 in pancreatic islets. The P1 and P2 promoter are involved in islet 11β-HSD1 transcription. It is concluded that 11β-HSD1 activity is upregulated by free fatty acids, a proinflammatory cytokine and is inhibited by high glucose, and by insulin under basal glucose conditions. Further studies will be necessary to investigate the role of other factors which are also altered in obesity and diabetes, and perhaps more importantly, effects of the factors in combination. In addition, detailed mechanistic links between the regulatory factors identified in this chapter and the molecular control of specific transcription factors at the P1 and P2 promoters will be important to explore.

Chapter 4

β-cell-specific Overexpression of 11β-HSD1 Reverses High-fat Diet Induced β-cell Failure

4 β-cell-specific overexpression of 11β-HSD1 reverses high-fat diet induced β-cell failure

4.1 Introduction

4.1.1 Type 2 diabetes and the role of the β-cell

Diabetes has become a worldwide disease of 'epidemic' proportions (Wild et al. 2004). Type 2 diabetes (T2D) is characterised by apoptotic β-cell death and a lack of responsiveness of the β-cell to secrete appropriate amounts of insulin after a meal. This usually occurs after a prolonged period of insulin resistance; a generalised lack of responsiveness of glucose consumption and storage in tissues (Martin et al. 1992). Furthermore, T2D often occurs in parallel with obesity and metabolic syndrome (Huang et al. 2009) suggesting the pathogenesis of insulin resistance originates at least in part from excessive adipose tissue or its dysfunction.

The β-cell is remarkable in its ability to synthesise and release insulin in a very precise manner to maintain glucose homeostasis (Described in 1.3.2). Changes in glucose levels are the main regulator of insulin secretion and are exquisitely sensed across a physiological range by β-cells (Maechler et al. 2006). In T2D, a period of prolonged hyperglycaemia caused by muscle and liver insulin resistance initially provokes increased insulin secretion to maintain normal glycaemia (β-cell compensation). Rodent studies show two important mechanisms, expansion of the β-cell mass (Steil et al. 2001; Liu et al. 2002; Jetton et al. 2005) and enhanced β-cell function (Chen et al. 1994; Liu et al. 2002) are involved in this process. However, type 2 diabetes later ensues due to a progressive deterioration in β-cell function (Jetton et al. 2005) associated with β-cell loss by apoptosis (β-cell failure) (Butler et al. 2003; Yoon et al. 2003). Recent genetic studies in large human populations

(genome wide association) have begun to identify several candidate genes of diabetes, the majority of which are associated with β-cell function, such as DGKB/TMEM195 and MADD which contribute to β-cell mass and insulin secretion (Dupuis et al. 2010).

4.1.2 11β-HSD1 in metabolic disorders

Excess plasma GCs (described in 1.4.3.3) exacerbate hyperglycaemia (Larsson and Ahren 1996) and insulin resistance (Sakoda et al. 2000; Smith and Muscat 2005), as evidenced by rare conditions such as Cushing syndrome. However, during the more prevalent idiopathic human metabolic disease development, elevated tissue 11β-HSD1, which mainly converts inactive GC (cortisone, 11β-dehydrocorticosterone) to active GC (cortisol, corticosterone) (Bush et al. 1968; Seckl and Walker 2001), rather than increasing circulating GC levels (Morton and Seckl 2008) appears to underlie the pathogenesis. This has been supported by several transgenic mouse models. Transgenic overexpression of 11β-HSD1 in adipose tissue caused visceral obesity, insulin resistance, type 2 diabetes and hypertension in mice (Masuzaki et al. 2001) recapitulating the elevation of adipose 11β-HSD1 observed in human obesity and metabolic syndrome (Morton and Seckl 2008). A high level of 11β-HSD1 in liver leads to mild insulin resistance and dyslipideamia (Paterson et al. 2004), which mimicked perhaps rarer instances of metabolic disturbance. Conversely, 11β-HSD1 knock out mice proved to be resistant to high fat diet induced type 2 diabetes, and had increased hepatic and adipocyte insulin sensitivity (Morton et al. 2001; Morton et al. 2004), an observation that has also been indicated by pharmacological studies using 11β-HSD1 inhibitors (Walker et al. 1995; Livingstone et al. 2000; Alberts et al. 2002; Barf et al. 2002; Alberts et al. 2003; Hermanowski-Vosatka et al. 2005; Webster et al. 2007; Boyle 2008; Bujalska et al. 2008; Boyle and Kowalski 2009; Rosenstock et al. 2010).

4.1.3 The effects of glucocorticoids and 11β-HSD1 on insulin secretion

A high level of 11β-HSD1 activity was found in pancreatic islets from obese diabetic rodent models (Duplomb et al. 2004; Ortsater et al. 2005). Incubation of islets from obese ($Lep^{ob/ob}$) mice with the 11β-HSD1 substrate 11DHC suppressed GSIS *in vitro* (Davani et al. 2000; Ortsater et al. 2005), leading the authors to hypothesise that high islet 11β-HSD1 was suppressive for GSIS. However, incubation of islets from $Lep^{ob/ob}$ with relatively low 5nM 11DHC showed an initial stimulatory effect on GSIS (Ortsater et al. 2005). These findings open up a wider controversy in the literature concerning the effects of GCs on islet function. Thus, although several studies have investigated GCs effects on β-cell GSIS, inconsistencies due to different species and methods are found (details in 1.4.3.3.4). In general, high-dose and long-term GCs exert inhibitory effects on GSIS *in vitro* (Billaudel and Sutter 1979; Barseghian et al. 1982; Pierluissi et al. 1986; Gremlich et al. 1997; Lambillotte et al. 1997; Davani et al. 2000; Jeong et al. 2001; Ortsater et al. 2005; Swali et al. 2008); while low-dose (Ortsater et al. 2005) or short-term GCs increase (Hult et al. 2009) and physiological concentration of GCs have no effect on GSIS (Jeong et al. 2001). Isolated islets from dexamethasone treated rats display an increase of GSIS (Karlsson et al. 2001; Choi et al. 2006; Rafacho et al. 2008; Rafacho et al. 2010). In contrast in mice, GCs reduce GSIS *in vivo* (Khan et al. 1992; Delaunay et al. 1997; Ling et al. 1998). The consequence of chronic elevation of 11β-HSD1 activity on β-cell function was therefore not straight forward to predict, although one could aruge the weight of evidence was for an inhibitory action of GCs on GSIS.

Given that inhibition of 11β-HSD1 is now advocated as a therapeutic treatment for obesity (reviewed in Hughes et al. 2008), we aimed to more fully understand the relationship between β-cell 11β-HSD1 and β-cell function. To do this we created a β-cell-specific 11β-HSD1 overexpression model in the C57BL/KsJ mouse genetic background strain that was prone to develop β-cell failure when fed high fat diets (Korsgren et al. 1990).

4.2 Hypothesis

We hypothesized that increased glucocorticoid action mediated by increased 11β-HSD1 expression may accelerate hyperglycaemia and diabetes by amplifying the inhibitory effects of glucocorticoids in β-cells.

4.3 Aims

1. To validate the β-cell specific 11β-HSD1 overexpression model.
2. To test the effects of β-cell specific 11β-HSD1 overexpression on a diet-induced T2D *in vivo* and *in vitro*.
3. To determine the effect of diet on expression of islet 11β-HSD1 in models with varying susceptibly to β-cell failure.

4.4 Experimental design

4.4.1 Creation of a β-cell-specific 11β-HSD1 overexpressing transgenic mouse model

As described in 2.1.1, the β-cell specific 11β-HSD1 overexpression mice model was created by using a β-cell specific mouse insulin promoter (MIP, 8.3kb) (Hara et al. 2003) from *Hind*III (-8280) and Not I (+12) upstream of the rat 11β-HSD1 cDNA (1.265bp) containing *EcoR*I fragment. The construct-mediated 11β-HSD1 activity and its glucose-inducibility was tested in clonal β-cells *in vitro* (Turban et al. unpublished), micro-injected into blastocysts from the C57BL/KsJ line and implanted in pseudo-pregnant females. Control C57BL/KsJ mice were bred in house. The microinjection was performed by the University of Edinburgh GIST Unit.

4.4.2 Genotyping of MIP-HSD1 transgenic mice

The genotype was determined by extracting the genomic DNA from mice tail tips (Described in 2.8.1), followed by PCR using primers specific to the upstream MIP promoter and an internal sequence of the rat cDNA transgene, which includes one diagnostic EcoR I site which we used to confirm transgene integration. This PCR product (650bp) was digested by restriction enzyme EcoR I to give two bands at 430bp and 220bp which were resolved in a 2% agarose gel. The MIP-HSD1 plasmid vector was used as a positive control (Described in 2.8.2).

To determine copy number, genomic DNA was digested with the infrequent cutter Afl III (the enzyme cuts at 6.897kb of the MIP-r11β-HSD1 transgene) followed by Southern blotting was performed with the rat 11β-HSD1 gene cDNA (1.265kb) as a probe to recognize both the endogenous and transgenic bands. Signal intensity was used to determine copy number to distinguish the heterozygous and homozygous mice. We used this method to screen all the generations of transgenic mice

(Described in 2.8.3).

4.4.3 11β-HSD1 activity in MIP-HSD1 transgenic mice

11β-HSD1 possesses both reductase and dehydrogenase activities (Low et al. 1994; Jamieson et al. 1995). As we have found that the reductase activity of 11β-HSD1 is predominant in pancreatic islets (Described in chapter 3), it was essential to find out whether reductase remains the main activity with overexpression in transgenic MIP-HSD1 mice, and whether it is glucose inducible as a result of being linked with the insulin promoter regulatory elements. Batches of 100 islets were isolated from KsJ and MIP-HSD1 mice and incubated with [^{3}H] labelled 11-dehydrocorticosterone (A compound) to measure reductase activity; while [^{3}H] labelled corticosterone (B compound) was incubated to test the dehydrogenase activity. All islets were incubated in either 2.8mM low glucose concentrations or 16.8mM high glucose concentrations. Activities were measured by TLC methods (Described in 2.9).

4.4.4 β-cell specificity of MIP-HSD1 transgene expression

To further investigate whether the 11β-HSD1 transgene was highly expressed within the β-cell of MIP-HSD1 transgenic mice, KsJ control and MIP-HSD1$^{tg/+}$ mice pancreas was stained with 11β-HSD1 antibody by immunohistochemistry.

4.4.5 The effect of the MIP-HSD1$^{tg/+}$ transgene on intraperitoneal glucose tolerance

GTT is widely used to determine how quickly glucose can be cleared from the blood after glucose administration. IpGTT was performed as described in 2.3.1. Insulin evolution curves (an indicator of β-cell responsiveness to glucose load *in vivo*), were also determined. Both KsJ and MIP-HSD1$^{tg/+}$ mice were on chow diet, and age-matched.

4.4.6 Induction of glucose intolerance with chronic high fat feeding

To induce glucose intolerance, mice were fed a hypercaloric (high-fat) diet (58 kcal% fat w/sucrose Surwit Diet) (Surwit et al. 1998) for 12 weeks, or a low fat control diet (11 kcal% fat w/cornstarch Surwit Diet). Body weight was measured every three days and fat mass, pancreas and liver mass were measured at necropsy.

4.4.7 Assessment of plasma corticosterone level in MIP-HSD1 mice

To find out whether MIP-HSD1 transgenic mice have altered at the plasmacorticosterone level. The mice were single-housed and after a two-day acclimation, blood samples were taken at the nadir (8am) by venesection within 1min of disturbing the animal to avoid confounding elevation of corticosterone stress-responses (≥1min in mice). Plasma corticosterone was measured by a RIA method using ^{3}H-corticosterone label and a polyclonal anti-corticosterone antibody (Described in 2.6.4).

4.4.8 Investigation of islet insulin secretion *in vivo*

To more rigorously test whether chronic elevation of β-cell 11β-HSD1 affected the islet GSIS of MIP-HSD1 transgenic mice *in vivo*, intravenous glucose tolerance test (ivGTT) was used. This method can be used to more closely monitor early first-phase (1 to 3 minutes) as well as second phase of insulin secretion after glucose challenge compared to intraperitoneal GTT and oral GTT which can exhibit marked variation in time of glucose absorption, especially in obese compared to lean animals (Cobelli et al. 2007). Furthermore, by applying a continuous bolus over a one hour period independently of glucose uptake, we were able to assess continuous and maximal β–cell responses. As described in 2.3.2, mice were administered glucose intravenously and plasma glucose level was measured by glucose meter (Details in 2.1.6.1). Blood samples were collected for further insulin ELISA assay (Details in 2.1.6.2).

4.4.9 Investigation of MIP-HSD1 islet insulin secretion *in vitro*

The islet autonomous insulin secretion was measured, as it is a way of assessing islet function independent of potentially altered islet number. Islets were isolated from all genotypes (Details in 2.7), and ten comparably sized islets were cultured in one well of a 24-well plate. High glucose (25mM) was used to stimulate islet insulin secretion (Details in 2.10). Medium was collected to measure insulin concentration by RIA (Details in 2.6.3).

4.4.10 The effects of high fat diet on islet 11β-HSD1 activity

Unlike the C57BL/KsJ strain, C57BL/6J mice are an obesity-prone model that can sustain a high level of insulin secretion to compensate for hyperglycaemia (Korsgren et al. 1990). C57BL/6J is widely used for metabolic studies and is the genetic background strain of the 11β-HSD1 knock out mice (Kotelevtsev et al. 1997; Morton et al. 2001; Morton et al. 2004). To begin to explore the concept that strain differences in islet 11β-HSD1 expression might be related to the β-cell compensatory capacity in response to high fat diet-induced insulin resistance, we compared the islet 11β-HSD1 activity levels in KsJ (non-compensating) with C57BL/6J (robustly compensating) after 12 weeks of high fat diet.

4.4.11 11β-HSD1 activity in islets from diabetic $Lep^{db/db}$ mice

Pancreatic islet 11β-HSD1 expression and activities were elevated in some obesity/diabetic rodent models (Duplomb et al. 2004; Ortsater et al. 2005). Another commonly used diabetic rodent model, $Lep^{db/db}$ mice, had increased 11β-HSD1 activity in liver (Liu et al. 2005) but this has not been addressed in islets. Islets were extracted from the appropriate KsJ wide type mice and $Lep^{db/db}$ mice. Batches of 100 islets per well were incubated at normal glucose concentration for 24 hours. TLC methods were used to measure the 11β-HSD1 activity.

4.5 Results

4.5.1 Creation of a β-cell specific 11β-HSD1 overexpressing transgenic mouse

MIP-HSD1 transgenic mice were made in-house using the mouse insulin promoter upstream of the rat cDNA 22 founder mice were viable after KsJ blastocyst injection into pseudopregnant females. 4 mice were found to be positive for the transgene but only one transmitted it to the next generation. Genomic DNA was extracted from the tail of each mouse and used to determine its genotype (as seen in figure 4-1, 4-2).

4.5.2 Genotyping of MIP-HSD1 transgenic mice

4.5.2.1 Identification of MIP-HSD1 transgenic mice by PCR

As shown in Figure 4-1, KsJ mice presented no band at 650bp, or the predicted bands after enzyme digestion, while MIP-HSD1 transgenic mice showed the same specific bands as the MIP-HSD1 plasmid vector positive control.

4.5.2.2 Distinguishing heterozygous and homozygous MIP-11β-HSD1 transgenic mice by Southern blot

As shown in Figure 4-2 the lower three bands are the wild type (endogenous 11β-HSD1) bands, present in DNA of all mice. The upper four bands are the transgenic bands which are specific to DNA of transgenic mice after Afl III digestion, heterozygous 11β-HSD1 (MIP-HSD1$^{tg/+}$) had approximately 2-fold the gene dose and homozygous (MIP-HSD1$^{tg/tg}$) were nearly 4-fold (Figure 4-2).

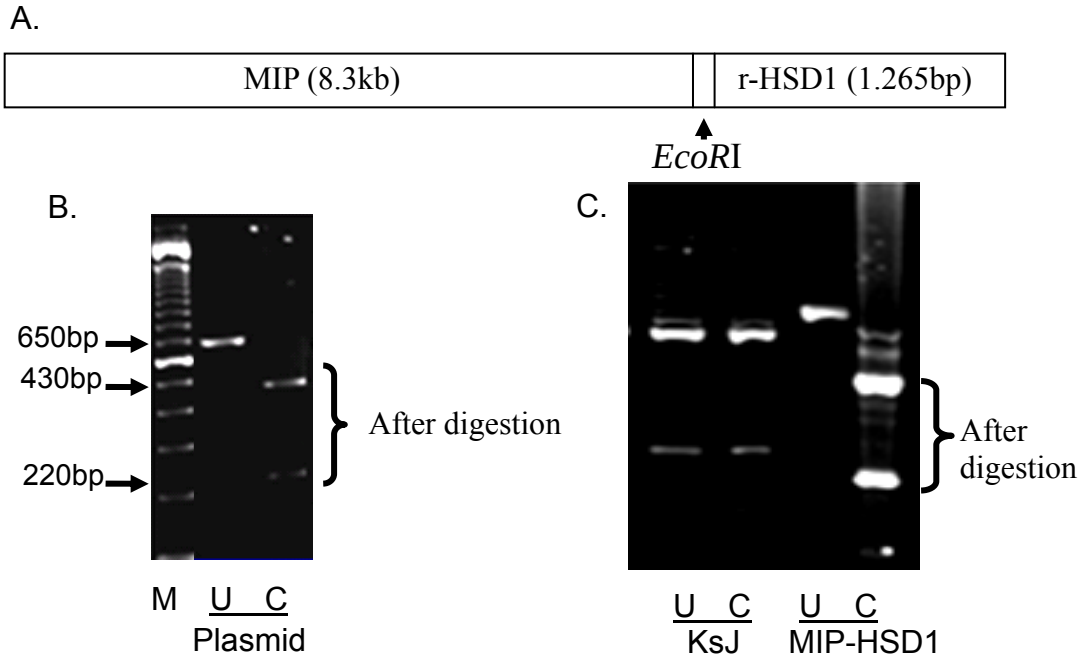


Figure 4-1. Genotyping MIP-HSD1 transgene by PCR. A. A schematic diagram of the MIP-HSD1 transgene. B. *EcoR*I digestion of the MIP-HSD1 plasmid control with characteristic restriction fragment pattern. C. The restriction fragment pattern observed after *EcoR*I digestion of KsJ genomic DNA and MIP-HSD1 genomic DNA. U-uncut, C-cut, M-100 base pair ladder.

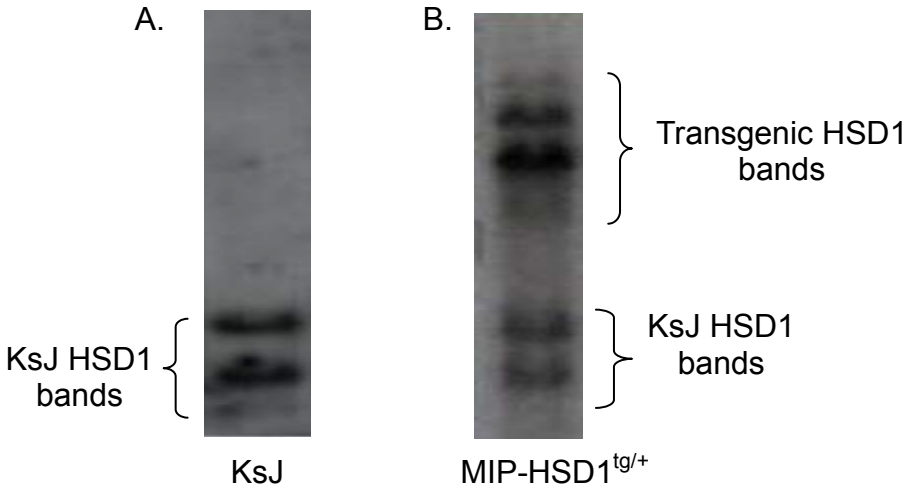


Figure 4-2. Genotyping transgenic mice by Southern. A. Southern blot pattern of KsJ wild type showing three lower bands of genomic 11β-HSD1. B. Southern blot pattern showing transgenic MIP-HSD1$^{tg/+}$ which also shows the upper four transgenic 11β-HSD1 bands.

4.5.3 11β-HSD1 activity in MIP-HSD1 pancreatic islets

4.5.3.1 11β-HSD1 reductase activity in MIP-HSD1 islets

Islets were isolated from KsJ (n=7), MIP-HSD1$^{tg/+}$ (n=5) transgenic mice and MIP-HSD1$^{tg/tg}$ (n=4). Reductase activity of 11β-HSD1 was measured after 24h incubation in media at low glucose or high glucose concentration in order to activate the insulin promoter which should drive increased transcription of 11β-HSD1. As expected 11β-HSD1 reductase activity and induction with glucose was higher in MIP-HSD1 transgenic mice with a gene-dose-dependent increase in the MIP-HSD1$^{tg/tg}$ mice. The reductase activity of 11β-HSD1 was not glucose inducible in KsJ mice *in vitro* (Figure 4-3).

4.5.3.2 11β-HSD1 dehydrogenase activity is low in MIP-HSD1$^{tg/+}$ islets

11β-HSD1 is a bidirectional enzyme, so the dehydrogenase activity of 11β-HSD1 was also measured in KsJ and MIP-HSD1$^{tg/+}$ mice. There is little dehydrogenase activity of 11β-HSD1 in islets. After 24h incubation in media at low glucose or high glucose concentration, as expected 11β-HSD1 dehydrogenase activity was near 10 fold lower compared to the reductase and there was no differences between MIP-HSD1$^{tg/+}$ mice and the KsJ mice. Further the dehydrogenase activity of 11β-HSD1 was not glucose inducible. (Figure 4-4)

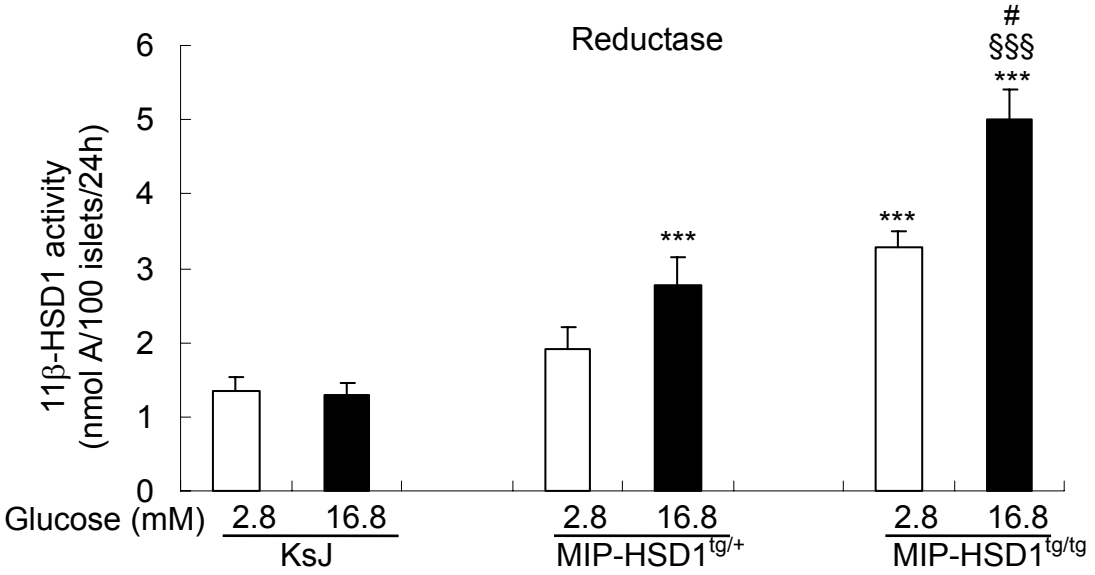


Figure 4-3. 11β-HSD1 reductase activity in different genotypes and glucose conditions. MIP-HSD1 11β-HSD1 reductase activity is increased and is glucose inducible. Values represented the mean ± S.E.M, *** P<0.001, (* versus KsJ 16.8mM glucose), §§§ P<0.01 (§ versus MIP-HSD1$^{tg/+}$), # P<0.05 (# versus MIP-HSD1$^{tg/tg}$ 2.8mM glucose), (One-way ANOVA, Tukey's Multiple Comparison Test), n=4-7.

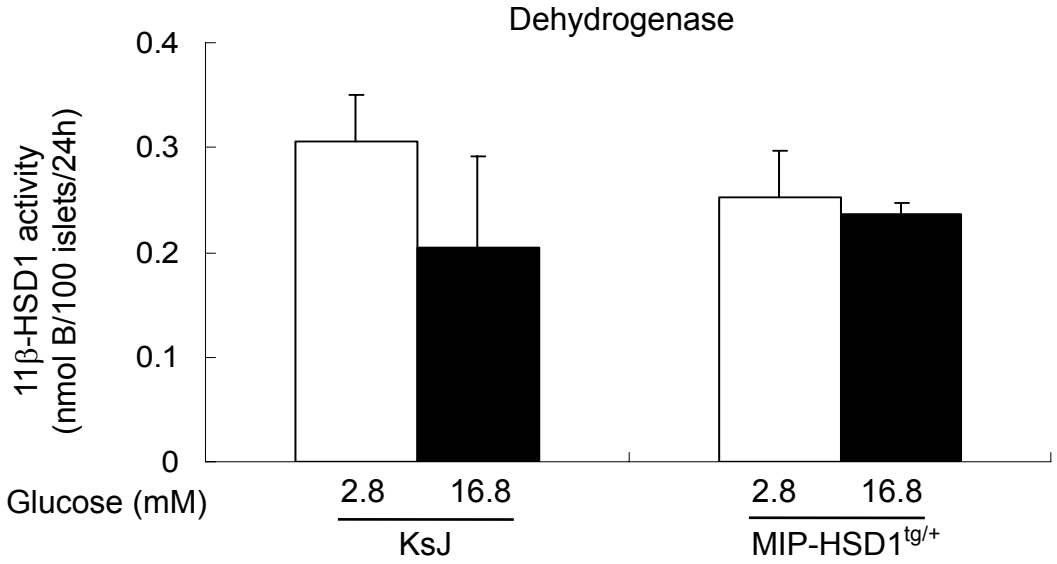


Figure 4-4. 11β-HSD1 dehydrogenase activity of KsJ and MIP-HSD1$^{tg/+}$ in different glucose conditions. 11β-HSD dehydrogenase activity, which is ten fold lower than reductase, was comparable in both genotype, and not affected by glucose. Values represented the mean ± S.E.M, not significant, One-way ANOVA, Tukey's Multiple Comparison Test, n=4-6.

4.5.4 Basal insulin secretory profile of MIP-HSD1$^{tg/+}$ mice

IpGTTs (2g/kg glucose) were performed on KsJ and heterozygous MIP-HSD1$^{tg/+}$ mice. Analysis of the glucose tolerance tests (Figure 4-5) showed similar increases of plasma glucose level in response to glucose injection between both groups. Moreover the glucose disposal represented by disappearance of blood glucose over the time, was comparable between KsJ and MIP-HSD1$^{tg/+}$ mice. Similarly plasma insulin concentrations rose normally after the increase of glucose in the blood and decreased at a normal rate while the body cleared the glucose (Figure 4-6).

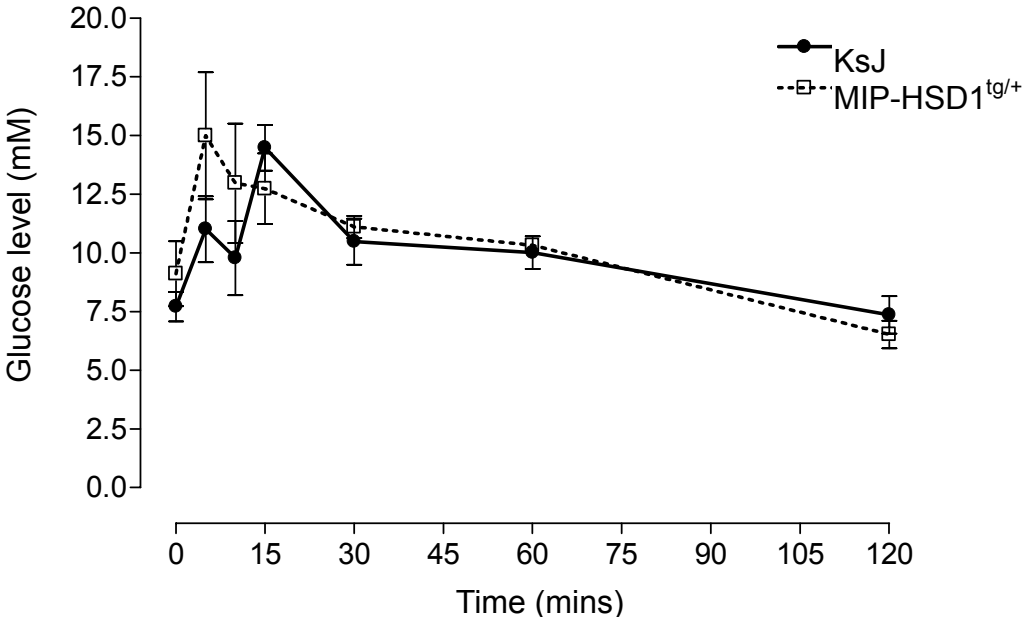


Figure 4-5. IpGTT plasma glucose levels for KsJ and MIP-HSD1$^{tg/+}$. The glucose load was administered shortly after time 0, and blood was sampled at 0, 5, 10, 15, 30, 60 and 120min. Values represent the mean ± S.E.M, Repeated measures ANOVA, n=6, not significant.

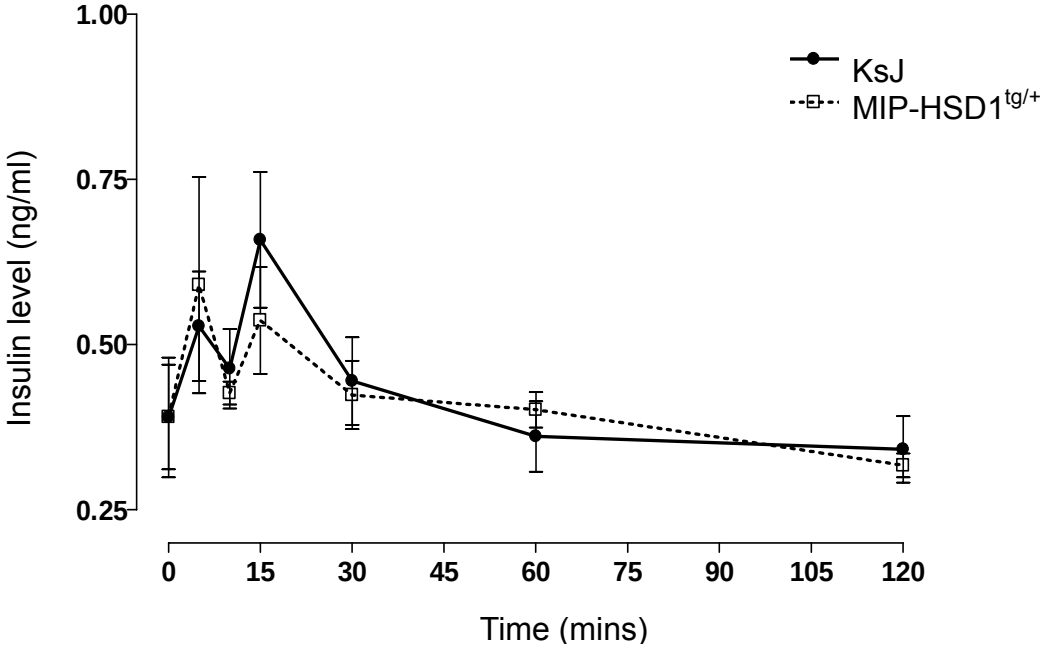


Figure 4-6. IpGTT plasma insulin levels for KsJ and MIP-HSD1$^{tg/+}$. The glucose load was administered at shortly after time 0, and blood sampled at 0, 5, 10, 15, 30, 60 and 120min. Values represent the mean ± S.E.M, Repeated measures ANOVA, n=6, not significant.

4.5.5 The effects of high fat feeding on MIP-HSD1 mice

Physiological characteristics of MIP-HSD1 mice were determined after 12 weeks of high fat feeding. MIP-HSD1 mice appeared grossly normal and the MIP-HSD1 mice showed a small but significant increase of weight gain (Table 4-1). The weight of the individual fat pads, pancreas and liver was not different between genotypes. Fasting plasma insulin and glucose was comparable between genotypes on chow diet and HF diet (Table 4-1).

Table 4-1. Physiological characteristics of MIP-HSD1 mice

	KsJ CD	KsJ HF	MIP$^{tg/+}$ CD	MIP$^{tg/+}$ HF	MIP$^{tg/tg}$ CD	MIP$^{tg/tg}$ HF
Body weight (g)	29.1±0.36	30.0±0.38	30.0±0.39	29.5±0.42	30.2±0.4	29.2±0.3
Weight gain (g)	0.21±0.31	1.66±0.28*	1.01±0.25	2.41±0.32*§	1.21±0.35	2.84±0.23$^{*\#}$
Epididymal mass (g)	0.53±0.06	0.93±0.07*	0.47±0.03	0.7±0.06	0.37±0.03	0.59±0.04
Pancreas (g)	0.29±0.04	0.30±0.04	0.34±0.02	0.29±0.05	0.28±0.01	0.29±0.02
Liver mass (g)	1.363±0.07	1.395±0.04	1.193±0.07	1.248±0.04	1.078±0.04	1.121±0.06
Fasting glucose (mM)	5.4±0.32	5.6±0.48	4.89±0.6	5.2±0.24	5.3±0.3	5.25±0.3
Fasting insulin (ng/ml)	0.85±0.33	0.48±0.27	0.56±0.11	0.74±0.33	0.56±0.35	0.43±0.17

Table 4-1. Physiological parameters of KsJ and MIP-HSD1 mice after high fat feeding for 12 weeks. Plasma and body weight data were collected on the first day, and at the end of the study, the tissues were collected. * $P<0.05$ (versus KsJ CD), § $P<0.05$ (versus MIP-HSD1$^{tg/+}$ CD), # $P<0.05$ (versus MIP-HSD1$^{tg/tg}$ CD). Values represented the mean ± S.E.M, One-way ANOVA, Tukey 's Multiple Comparison Test, n=6.

4.5.6 The effects of high fat feeding on plasma corticosterone levels in MIP-HSD1 mice

Plasma corticosterone levels were measured by RIA to determine if the MIP-HSD1 transgene effects, if any, might affect the HPA function. The plasma corticosterone level was comparable between the genotypes and diets (Figure 4-7).

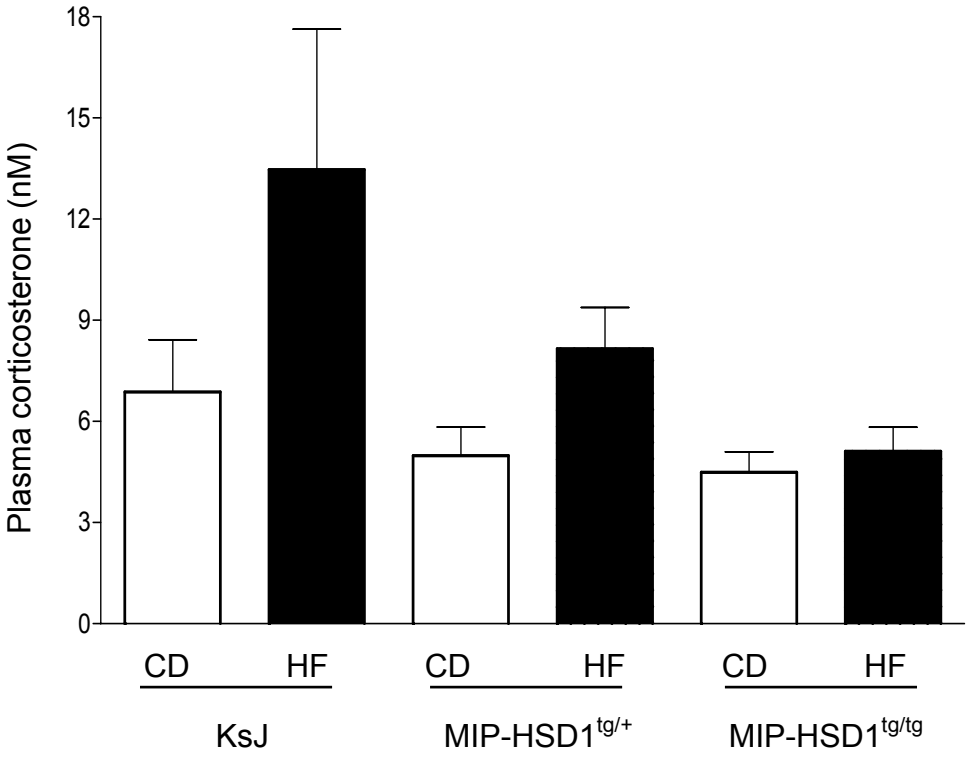
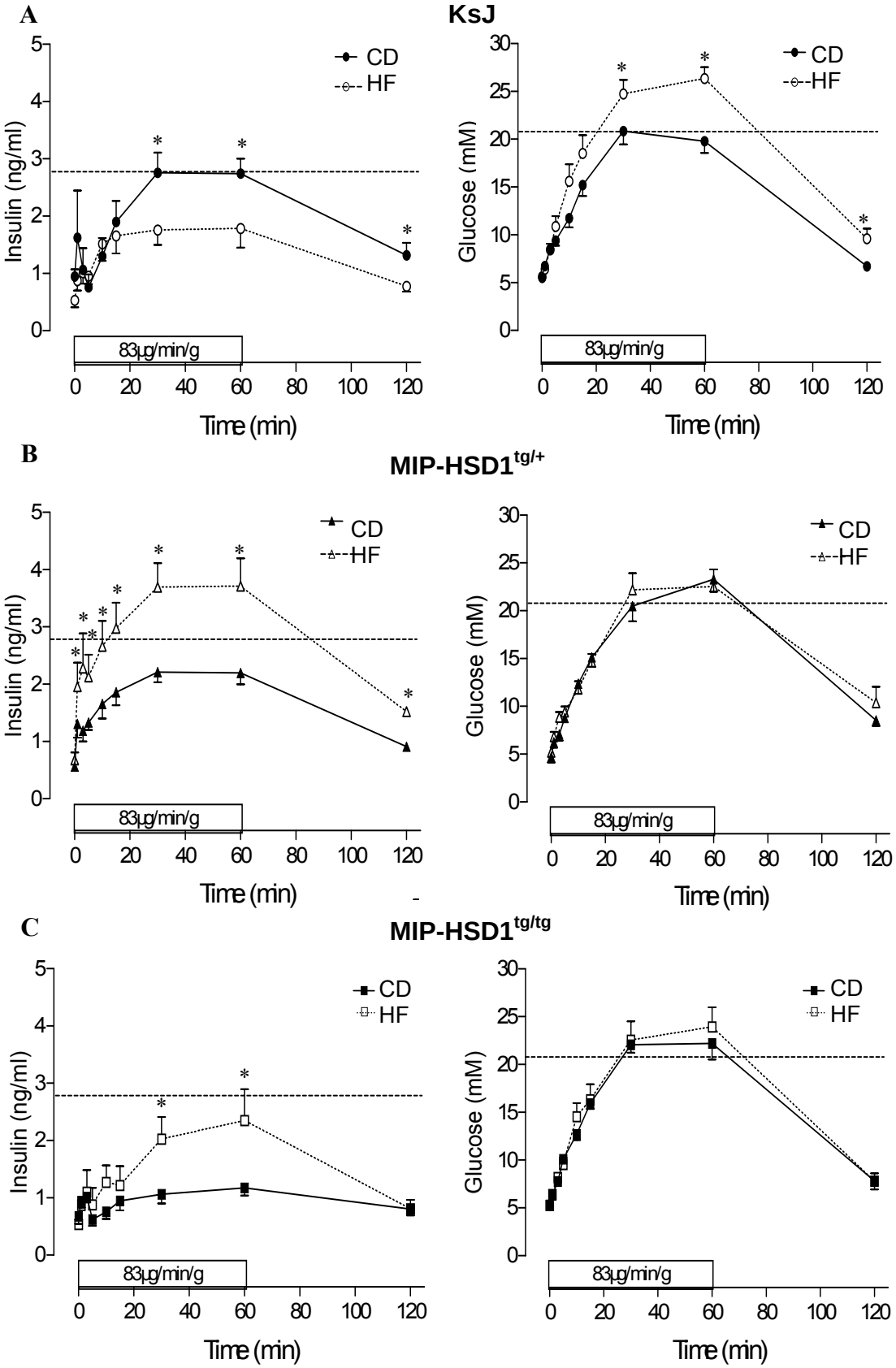


Figure 4-7. Plasma corticosterone levels in different genotypes and diets. No difference was observed between genotypes and diets. Values represented the mean ± S.E.M, (One-way ANOVA, Tukey's Multiple Comparison Test), n=6-12.

4.5.7 The effects of high fat feeding on MIP-HSD1 insulin secretion *in vivo*: Intravenous glucose tolerance

In order to investigate the potentially pathogenic effects of high β-cell 11β-HSD1 in diabetes, glucose intolerance and weight gain were induced in the mice by feeding a high fat diet for 12 weeks. A high glucose concentration (3mg/g BW, 25% glucose at a rate 0.83μg/min/g) was constantly infused intravenously to stimulate maximal insulin secretion for one hour with blood sampling at 0, 1, 3, 5, 10, 15, 30, 60, and 120mins in order to closely follow the first and second phases of insulin secretion. The KsJ high fat fed group secretes less insulin than KsJ chow diet and developed glucose intolerance after high dose glucose loading. However, the MIP-HSD1$^{tg/+}$ high fat fed group secreted significantly more insulin than MIP-HSD1$^{tg/+}$ chow diet fed and corrected glucose intolerance relative to HF fed KsJ mice as a result. MIP-HSD1$^{tg/tg}$ mice on both chow diet and high fat diet secreted significantly lower insulin than the KsJ control group during the first phase. However, MIP-HSD1$^{tg/tg}$ mice exhibited elevated insulin secretion compared to control diet fed MIP-HSD1$^{tg/tg}$ mice at second phase when fed a high fat diet. (Figure 4-8)

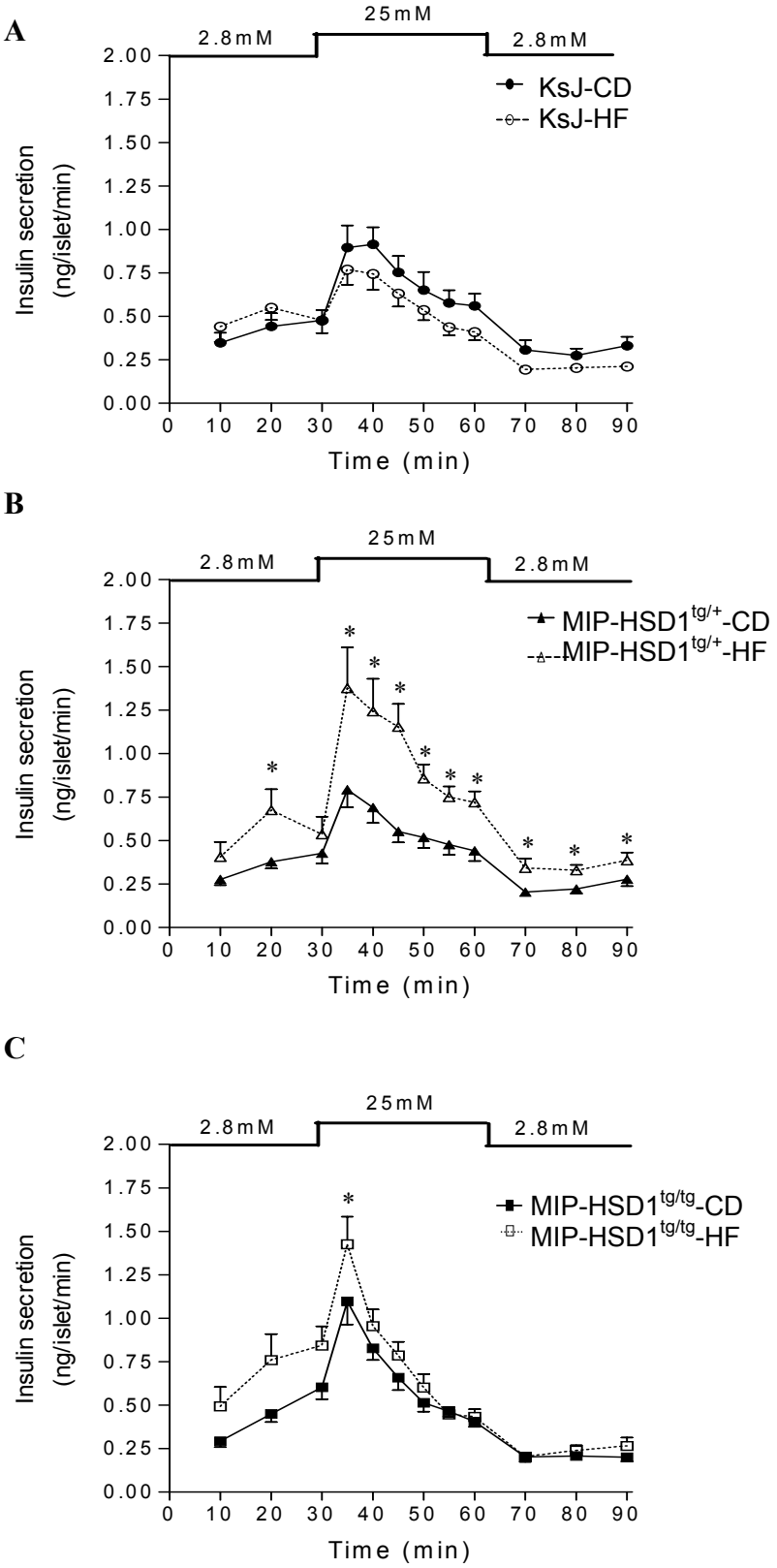
Figure 4-8. Plasma insulin and glucose levels in different genotypes and with different diets during LGTT. During one hour glucose loading, A. the plasma insulin levels for the high fat fed (HF) KsJ group secreted less insulin than chow diet fed (CD) KsJ group, whereas the glucose level was significantly higher in HF KsJ than CD KsJ mice. B. The plasma insulin level of the transgenic MIP-HSD1$^{tg/+}$ mice HF group secreted clearly more insulin in both phases compared to CD group, even higher than KsJ CD group, and glucose level was not different between HF and CD groups. C. In MIP-HSD1$^{tg/tg}$ HF group first phase insulin secretion was lost but increased in the second phase; the glucose level was similar between HF and CD. The broken horizontal lines represent the 2.8ng/ml plasma insulin level and the mean peak (~21mM) KsJ CD plasma glucose level for comparison across the groups. Values represent the mean ± S.E.M, * $P < 0.05$, Student t-test, n=6-8.


A
KsJ
CD
HF
Insulin (ng/ml)
Glucose (mM)
83µg/min/g
Time (min)
B
MIP-HSD1tg/+
C
MIP-HSD1tg/tg

4.5.8 The effects of high fat feeding on MIP-HSD1 islet insulin secretion in vitro

To confirm a direct β-cell specific effect of 11β-HSD1 overexpression on islet function, we looked at insulin secretion from isolated islets. Isolated islets from KsJ and transgenic mice were treated with 2.8mM glucose for 30mins, then 25mM for the following 30mins, which caused a peak of insulin secretion, after which islets were restored to 2.8mM glucose to test the reversibility of glucose-stimulated insulin secretion (GSIS). KsJ on HF showed no compensatory insulin secretion after glucose stimulation. MIP-HSD1$^{tg/+}$ on HF had a higher basal insulin secretion and showed higher insulin release compared to other groups after stimulation by a high glucose concentration. Insulin secretion from MIP-HSD1$^{tg/tg}$ HF group was higher than MIP-HSD1$^{tg/tg}$ CD group but this was sustained only for the first 20 minutes after high glucose stimulation, in contrast to the MIP-HSD1$^{tg/+}$ mice. (Figure 4-9).

Figure 4-9. Glucose stimulates insulin secretion *in vitro* differentially with genotype and diet. A. Insulin secretion of KsJ HF group was lower than KsJ CD group. B. Basal and high glucose stimulated insulin released was higher in MIP-HSD1$^{tg/+}$ HF group. C. MIP-HSD1$^{tg/tg}$ on HF diet showed an elevated basal insulin secretion but impaired second phase GSIS. Values represent the mean ± S.E.M, * P< 0.05, Student t-test, n=6.


A
2.8mM
25mM
2.8mM
KsJ-CD
KsJ-HF
Insulin secretion (ng/islet/min)
Time (min)
B
2.8mM
25mM
2.8mM
MIP-HSD1tg/+-CD
MIP-HSD1tg/+-HF
Insulin secretion (ng/islet/min)
Time (min)
C
2.8mM
25mM
2.8mM
MIP-HSD1tg/tg-CD
MIP-HSD1tg/tg-HF
Insulin secretion (ng/islet/min)
Time (min)

4.5.9 Differential islet 11β-HSD1 activity in mouse strains with varying susceptibility to β-cell failure

After 12 weeks of high fat diet, islets were isolated from C57BL/6J and KsJ mice to evaluate 11β-HSD1 activity. High fat diet increased 11β-HSD1 activity in islets of the compensation-competent C57BL/6J strain while islet 11β-HSD1 activity was decreased by high fat diet in islets of the β-cell failure susceptible KsJ mice. (Figure 4-10)

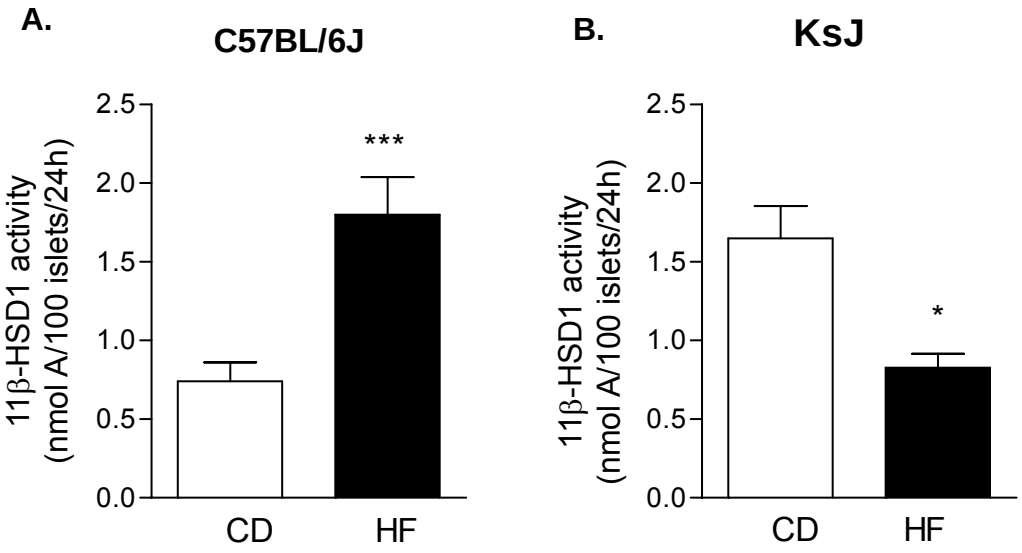


Figure 4-10. 11β-HSD1 activity of C57BL/6J and KsJ islets after high fat feeding. 11β-HSD1 activity significantly increased in C57BL/6J mice after 12 weeks high fat feeding (A), whereas there was a significant reduction in KsJ mice (B). Values represented the mean ± S.E.M, ***P<0.001, *P<0.05 Student t-test, n=6.

4.5.10 11β-HSD1 is markedly elevated in islets from diabetic Lep$^{db/db}$ mice

11β-HSD1 activity was increased in pancreatic islets from Lep$^{ob/ob}$ and Zuker (ZDF) rats where this was hypothesized to impair β-cell function (Duplomb et al. 2004; Ortsater et al. 2005). Having shown that reductase and not dehydrogenase predominates in islets, we wished to confirm whether this was altered in the diabetic model Lep$^{db/db}$ mice (See 3.3.1 and 4.5.3.2). Thus, the islet 11β-HSD1 reductase activity of KsJ control was compared to that of diabetic Lep$^{db/db}$ mice. Figure 4-11 presented a clear increase of pancreatic islet 11β-HSD1 reductase activity in Lep$^{db/db}$ mice.

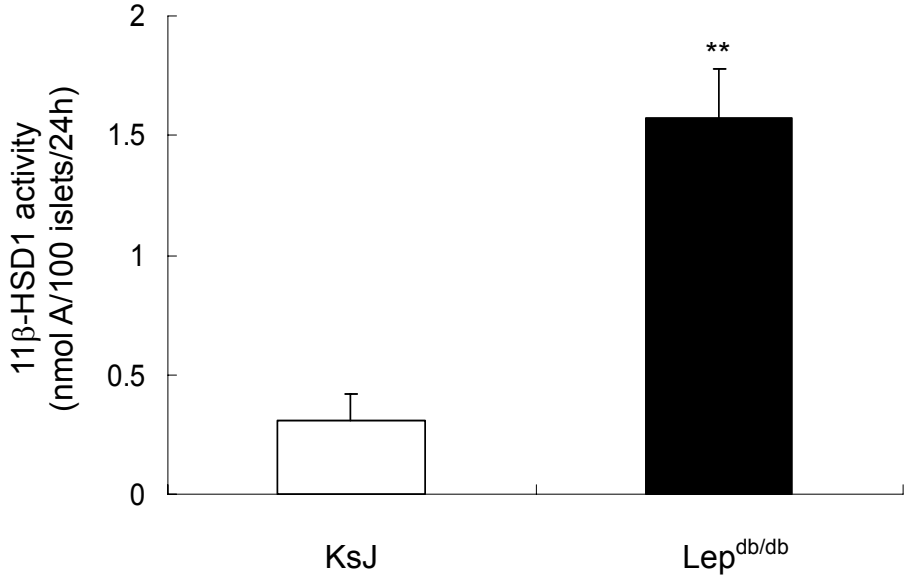


Figure 4-11. 11β-HSD1 reductase activity was significantly elevated in islets of the diabetic model Lep$^{db/db}$ mice. Islets were isolated from KsJ and Lep$^{db/db}$ mice. Values represent the mean ± S.E.M, ** P=0.0017, Student t-test, KsJ group n=4, Lep$^{db/db}$ group n=7.

4.6 Discussion

Unexpectedly, our transgenic model demonstrates that modestly elevated levels of β-cell specific 11β-HSD1 overexpression (heterozygote, MIP-HSD1$^{tg/+}$) improves β-cell function *in vitro* and *in vivo*, counteracting the glucose intolerance caused by high fat feeding. This suggests increased β-cell 11β-HSD1 is a part of the physiological adaptive compensatory response of islets during prediabetes, revealing for the first time a markedly different outcome other than the diabetogenic effects induced by overexpression of 11β-HSD1 in adipose tissue and liver (Masuzaki et al. 2001; Paterson et al. 2004). We have therefore rejected our original hypothesis, and must now refine our understanding of 11β-HSD1 action in β-cells. Homozygote transgenic mice (MIP-HSD1$^{tg/tg}$), which have a more pronounced increase in β-cell 11β-HSD1 levels, exhibit an attenuated second phase insulin secretory response compared to MIP-HSD1$^{tg/+}$ mice, even on normal chow diet. Surprisingly, this was adequate enough to maintain glucose tolerance, presumably due to sufficient efficacy of the lower insulin to act upon normally sensitive peripheral tissues such as muscle. MIP-HSD1$^{tg/tg}$ models a high expression of 11β-HSD1 as found in the diabetic Lep$^{db/db}$ islets and indicates that islet higher 11β-HSD1 expression might suppress β-cell insulin release. Previous studies have reported inconsistent results about GCs and/or 11β-HSD1 effects on islet insulin secretion, although most reports showed GCs either directly decreased islets GSIS of C57BL/6J (Swali et al. 2008) or showed this inhibitory effect indirectly by incubating islets with 11DHC, the 11β-HSD1 substrate in Lep$^{ob/ob}$ mice (Davani et al. 2000; Ortsater et al. 2005). A recent report showed that after short-term GC treatment islet GSIS was increased at first and second phase *in vitro* (Hult et al. 2009). Our data clarify the conflicting data in the literature suggesting there is an optimum level of 11β-HSD1 in β-cells which initially improves β-cell responses to glucose stimulation. Either a higher or lower level around this optimum level would suppresses β-cell GSIS, consistent with the inverted U-shaped dose response effects of GCs (Du et al. 2009). Thus, if the observations from our previous data can be extrapolated to the whole animal,

modestly elevated 11β-HSD1 in β-cells can ameliorate glucose intolerance. Beyond a protective threshold higher 11β-HSD1 expression (e.g. MIP-HSD1$^{tg/tg}$ and Lep$^{db/db}$) may worsen diabetes. These findings have profound implications for the potential use of 11β-HSD1 inhibitors clinically to prevent insulin resistance: these drugs may exacerbate diabetes if the β-cell effect outweighs peripheral insulin sensitisation.

11β-HSD1 is a bidirectional enzyme; previous results demonstrated 11β-HSD1 activity was predominantly reductase and was ten fold higher than dehydrogenase activity in islets of KsJ wild type mice (Showed in 3.4.1). Further increasing 11β-HSD1 in MIP-HSD1$^{tg/+}$ mice did not switch this, suggesting the H6PDH levels are sufficient to maintain GC amplification, presumably also when islets 11β-HSD1 is elevated in the disease state, as we found the Lep$^{db/db}$ mice.

The activity of islet 11β-HSD1 in the MIP-HSD1 model is glucose inducible, which means hyperglycaemia can manifest increased β-cell 11β-HSD1 activity during the development of diabetes. Although we had only one transgenic line, increasing the glucose concentration elevated the conversion of 11-dehydrocorticosterone to corticosterone in isolated islets from MIP-HSD1 transgenic mice with a gene dose effect, confirming the utility of our model as well as its glucose-responsiveness and suitability for studies modelling high β-cell specific 11β-HSD1 levels.

Our β-cell 11β-HSD1 overexpression model is therefore a functional and a valuable tool for determining the effects of β-cell 11β-HSD1 under pre-diabetic conditions. The MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ models the β-cell compensation or partial failure as found in a number of obesity and diabetes rodent models (Duplomb et al. 2004; Ortsater et al. 2005). Notably, since plasma GC levels were not significantly altered, the phenotype of the MIP-HSD1 transgenic mice must be due to β-cell intrinsic transgene specific effects.

IvGTT (LGGT) showed the KsJ mice on high fat diet developed glucose intolerance as a result of lower insulin output, ie. β-cell failure (reviewed in McGarry and

Dobbins 1999). This was confirmed by a lack of a compensatory response in the isolated islets. We further confirmed that MIP-HSD1$^{tg/+}$ mice and to a certain extent MIP-HSD1$^{tg/tg}$ mice derive islet autonomous protection *in vitro* through chronic elevation of β-cell 11β-HSD1. This is extremely important as it demonstrates that individual islets (β-cell) have altered function rather than there being altered peripheral glucose disposal and that the *in vivo* effect is not simply due to increased β-cell number through a potential developmental effect of the MIP-HSD1 transgene. Furthermore from the *in vitro* result, MIP-HSD1$^{tg/tg}$ high fat fed mice did not secrete as much insulin as MIP-HSD1$^{tg/+}$ when challenged by high glucose, which suggested a dose-dependent threshold for the protective effect of 11β-HSD1 expression in β-cell.

There are two ways for β-cell to compensate hyperglycaemia, one is by enhancing β-cell insulin secretory function (Chen et al. 1994; Liu et al. 2002), the other is expansion of β-cell mass (Steil et al. 2001; Liu et al. 2002; Jetton et al. 2005). As we have already shown MIP-HSD1$^{tg/+}$ mice had elevated autonomous islet function. Recent analysis of the pancreatic islet number by optical projection tomography (OPT) has shown increased islet number also (Turban et al. unpublished).

Pancreatic islet 11β-HSD1 activity was elevated in C57BL/6J mice after 12 weeks high fat diet exposure, whereas it was reduced in β-cell failure-prone KsJ mice, the genetic background of the MIP-HSD1 mice. MIP-HSD1$^{tg/+}$ mice show a comparable 11β-HSD1 activity to C57BL/6J mice that robustly compensate (with hyperinsulinaemia) for insulin resistance.

Others have shown that 11β-HSD1 activity mediated inhibition of islet insulin secretion (Davani et al. 2000; Swali et al. 2008). It has therefore been hypothesized that inhibition of 11β-HSD1 to decrease glucocorticoid activity in islets, will increased insulin release and protect against diabetes (Duplomb et al. 2004; Ortsater et al. 2005). Clinical studies using the prototypic drug, carbenoxolone (CBX), showed hepatic insulin sensitization in lean healthy subjects (Walker et al. 1995) and patients with type 2 diabetes (Andrews et al. 2003). It was also found that this

non-selective 11β-HSD1 inhibitor counteracted the suppressive effect of 11β-HSD1 substrate provision on isolated islets of $Lep^{ob/ob}$ mice (Davani et al. 2000; Ortsater et al. 2005), as did the selective inhibitor BVT. 2733 (Ortsater et al. 2005). However there were some conflicting results on whether there is an inhibitory effect of glucocorticoid on insulin secretion from isolated islets (Jeong et al. 2001; Hult et al. 2009) which may be explained by differences in concentration of glucocorticoid or duration of exposure in culture. Crucially we have found that modest, chronic elevation of β-cell 11β-HSD1 to levels found in robustly compensating (C57BL/6J) mice is protective against β-cell failure. The increase islet 11β-HSD1 in C57BL/6J mice indicates this is a physiological mechanism, indeed the first from our knowledge where a beneficial metabolic effect is derived from increased levels of the 11β-HSD1. However, the MIP-$HSD1^{tg/tg}$ and $Lep^{db/db}$ mice show that any compensatory mechanism may eventually overshoot and lead to a detrimental suppression of β-cell function. Dissecting the suppressive from the beneficial effects of β-cell 11β-HSD1 overexpression may lead to the identification of novel compensatory pathways that could be exploited therapeutically in diabetes.

Chapter 5

β-cell-specific Overexpression of 11β-HSD1 Reduces Hyperglycaemia in Streptozotocin-Induced Diabetes through Anti-inflammatory Effects

5 β-cell-specific overexpression of 11β-HSD1 reduces hyperglycaemia in streptozotocin-induced diabetes through anti-inflammatory effects

5.1 Introduction

Type 1 diabetes (T1D) is an autoimmune disease characterized by β-cell destruction. β-cell loss occurs through necrosis and apoptosis as a result of inflammation within islets (insulitis), which ultimately contributes to β-cell destruction. At early stages of T1D, inflammatory mediators induce and amplify the immune reaction against the β-cell. At later stages, inflammatory mediators suppress β-cell function, inhibit β-cell regeneration, stimulate peripheral insulin resistance and maintain insulitis (reviewed in Eizirik et al. 2009).

A well-established rodent model for type 1 diabetes is streptozotocin (STZ) treatment. STZ is β-cell toxic and because its molecular structure is similar to glucose (Figure 5.1) it is taken up selectively by the glucose transporter 2 (GLUT 2) which is highly expressed in the pancreatic β-cell. The mechanism of STZ induced β-cell death is related to its alkalising induced DNA damage. STZ also causes DNA methylation including N^7-methylguanine (7-MeG), N^3-methyladenine (3-MeA), and O^6-methylguanine (6-MeG) (reviewed in Bolzan and Bianchi 2002). Two experimental methods are commonly used to induce diabetes by STZ. The first model uses multiple low-dose STZ for 5 consecutive days in order to cause a mild β-cell loss and delayed-onset mild hyperglycaemia. In this model, β-cell death is mainly by apoptosis (O'Brien et al. 1996) due to accumulation of 3-MeA and/or 6-MeG (Glassner et al. 1999). The second uses a single high dose of STZ which causes almost complete β-cell death and a rapid onset of hyperglycaemia. Necrosis is a predominant cause of β-cell death in this model. In response to excess levels of

DNA adducts (3-MeA) by high dose of STZ, the Base Excision Repair (BER) pathway is over-activated and causes extensive activation of poly adenosine 5'diphosphate-ribose polymerase (PARP), rapid depletion of cellular Nicotinamide adenine dinucleotide (NAD^+) and cell death through necrosis (Murata et al. 1999). A robust inflammatory response is involved in both models (Huang and Taylor 1981).

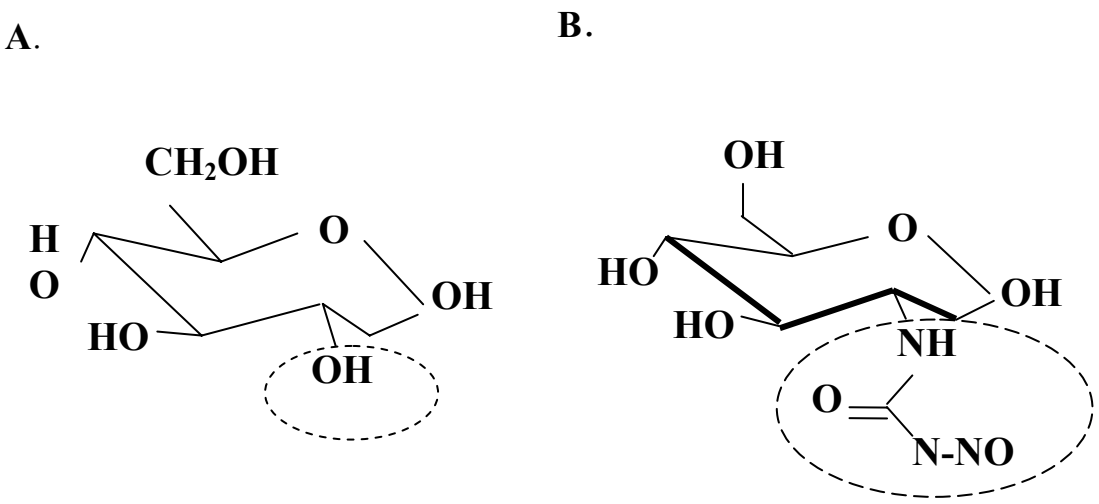


Figure 5-1. Structure of glucose and streptozotocin (STZ). The structures of glucose and STZ were determined by (Herr et al. 1967). Broken circle lines show the structural difference between glucose and STZ.

Under normal physiological conditions, the β-cell mass remains linear with body weight (Bonner-Weir 2000). β-cell mass is maintained through slow renewal and the β-cell half-life has been estimated at 30-60 days in young rats (Finegood et al. 1995). In humans stable β-cell mass is largely established by 20 years of age (Cnop et al. 2010). The slow rate of β-cell proliferation is balanced to compensate for their low level of turnover (Dor et al. 2004; Teta et al. 2007). In the pre-diabetic condition, hyperglycaemia increases both β-cell function (Chen et al. 1994) and islet mass in the short-term (Bonner-Weir and Smith 1994; Alonso et al. 2007). The adult islet mass expansion is predominantly through hypertrophy of existing β-cells (Jonas et al. 1999), β-cell proliferation (Dor et al. 2004; Teta et al. 2007), and neogenesis from progenitors also feature. Since there was a protective effect of β-cell specific 11β-HSD1 overexpression on β-cell number, function and survival with the low grade inflammation of type 2 diabetes, we were also interested to look at the numerous cell types proposed as possible pancreatic progenitors that could support generating new β-cell in the type 1 diabetes model. Thus, acinar cells (Zhou et al. 2008; Sangiorgi and Capecchi 2009); ductal epithelial cells (Bonner-Weir 2000; Xu et al. 2008); centroacinar cells (Rovira et al. ; Hayashi et al. 2003; Nagasao et al. 2003); mesenchymal-like nestin-expressing cells (Zulewski et al. 2001) and intra-islet δ cells (Fernandes et al. 1997) were considered. (Figure 5-2)

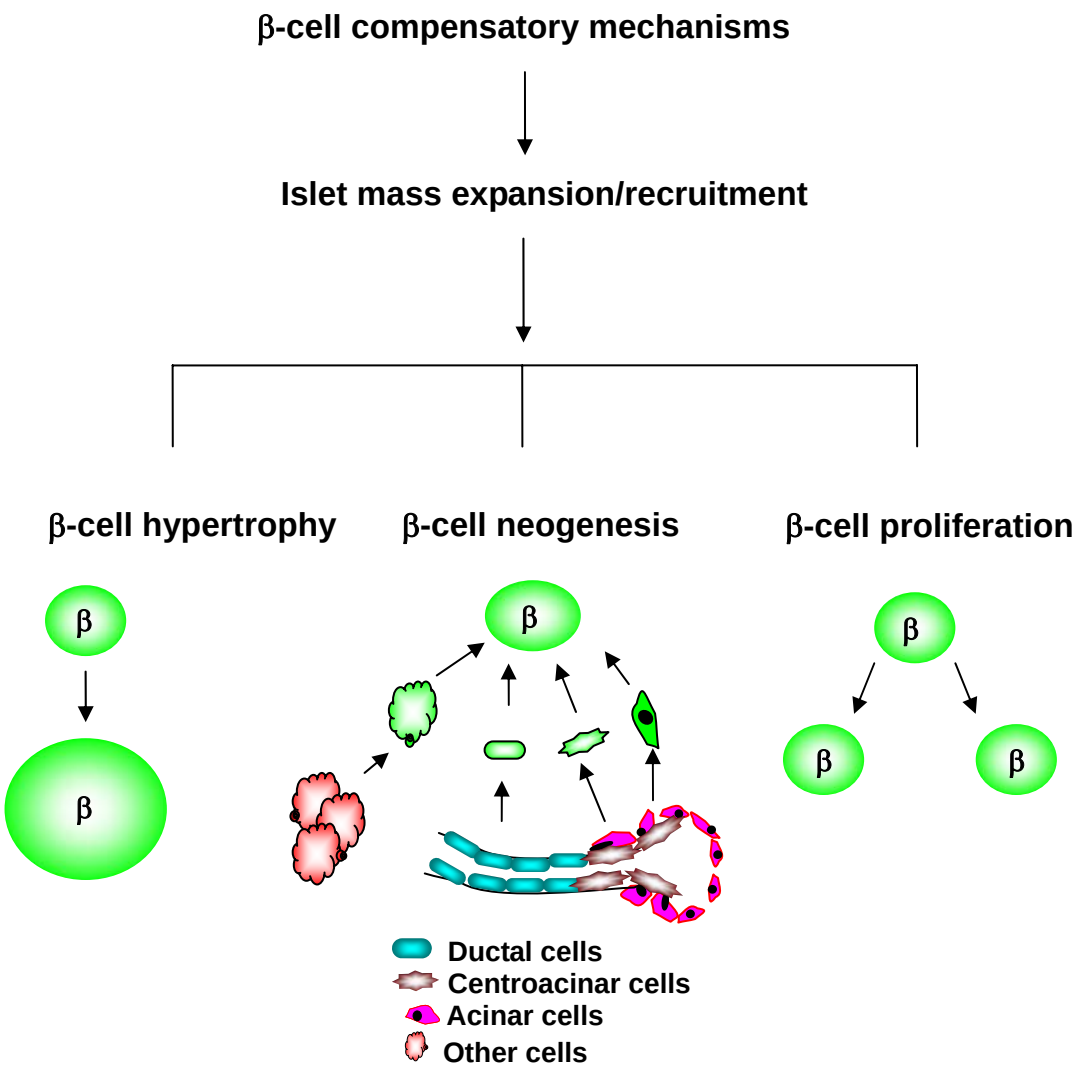


Figure 5-2. Possible ways for survival or recovery of β-cell mass after STZ-induced toxicity. STZ-induced β-cell loss and hyperglycaemia can be compensated by increasing islet β-cell hypertrophy; neogenesis, and β-cell proliferation from existing β-cells.

Besides the potential direct diabetogenic properties of GCs on β-cells and their promotion of peripheral insulin resistance (details in 1.4.3.3) (Lambillotte et al. 1997), GCs play an important part in pancreatic β-cell development (details in 1.4.3.1). Furthermore, GCs inhibit cell replication and induce differentiation by arresting the cell cycle at G1-phase *in vitro* (Rogatsky et al. 1997) in epithelial cells (Goya et al. 1993; Buse et al. 1995), fibroblasts (Frost et al. 1994), hepatoma cells (Cook et al. 1988; Sanchez et al. 1993), lung cancer cells (Greenberg et al. 2002) and pancreatic AR42J cells (Eum et al. 2003). Furthermore, overexpression of human 11β-HSD1 in osteosarcoma cells reduces their rate of replication (Rabbitt et al. 2002). And, high dose dexamethasone (1 and 0.5 mg/kg) and hydrocortisone (25mg/kg) increases rat β-cell replication *in vivo* (Rafacho et al. 2009; Jorns et al. 2010; Rafacho et al. 2010). In β-cells *in vitro*, GCs can induce β-cell apoptosis (Ranta et al. 2006). Administration of hydrocortisone (25mg/kg) *in vivo* increases apoptosis, counteracting stimulated proliferation of rat β-cell (Jorns et al. 2010). However, contrasting evidence showed that dexamethasone reduced β-cell apoptosis in the presence of cytokines *in vitro* (Chou et al. 2010). Thus it remains unclear what the dominant and direct effect of altered β-cell glucocorticoid action has on the progression of type 1 diabetes.

GCs are well known for their immunosuppressive and anti-inflammatory effects (Described in 1.4.3.2). This included increased macrophage-mediated ingestion of apoptotic leukocytes (Liu et al. 1999), induction of monocytes into highly phagocytic anti-inflammatory macrophages (Giles et al. 2001; Heasman et al. 2003), suppression of a number of transcriptional factors expressed in macrophages such as NF-κB, AP-1 and STAT families to alter the immune response (De Bosscher and Haegeman 2009), and regulation of the Th1/Th2 balance (Kovalovsky et al. 2000). GCs may therefore improve islet function by suppressing local inflammation (Figure 5-3), as suggested by others who used dexamethasone treatment in islets *in vitro* (Hult et al. 2009). Clearly, the dose and duration of GC exposure is important as demonstrated in Chapter 4.

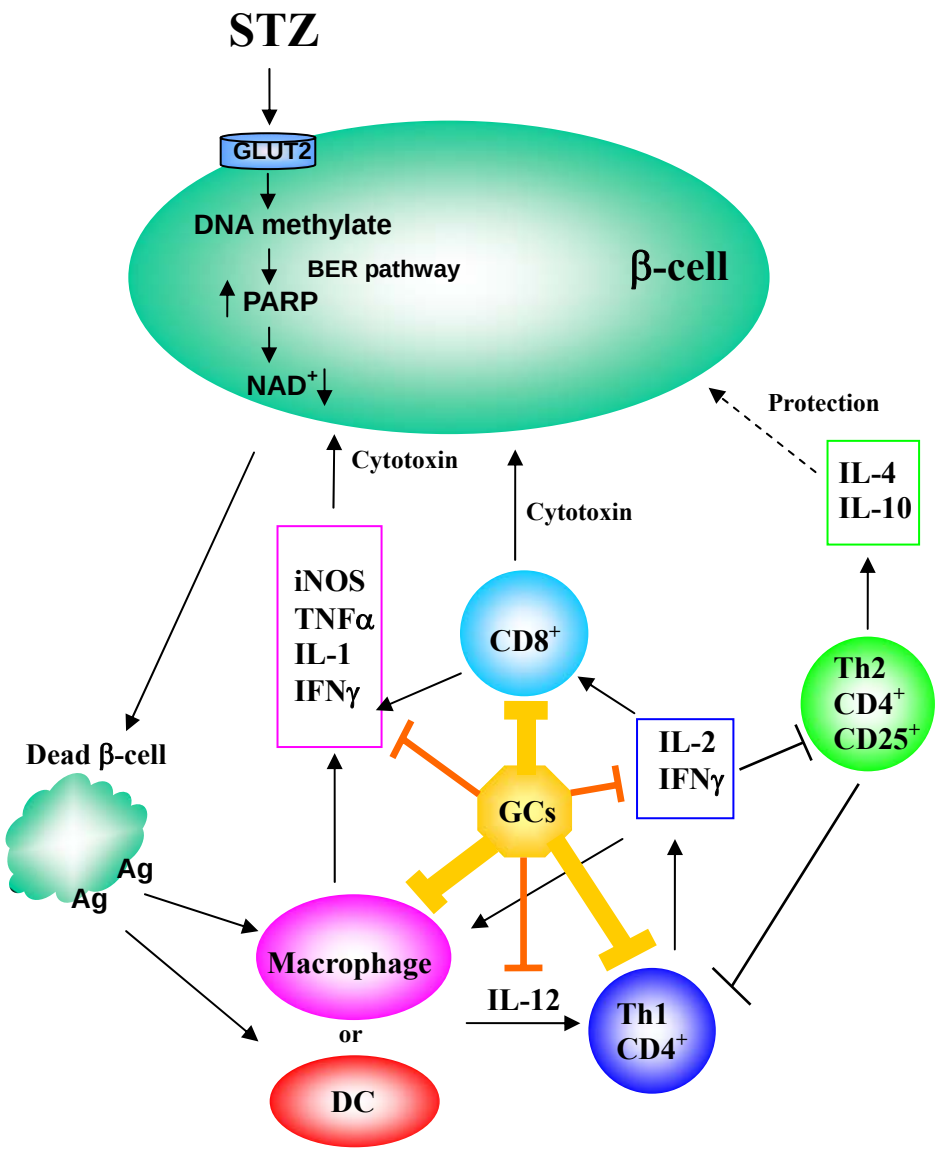


Figure 5-3. Regulatory influence of GCs on immune cells and cytokines found in STZ-induced diabetes. PARP increases through the BER pathway to repair adduct DNA damage caused by the alkylating effects of STZ which is taken up by GLUT2. The overactivated PARP rapidly depletes intracellular NAD^+ leading to β-cell destruction. The dying β-cells and the antigen (Ag) released from them are perceived by dendritic cells (DC) or macrophages to secrete IL-12 that activates $CD4^+$ Th1 cells. The active Th1 cells produce IL-2 and IFNγ which inhibit $CD4^+$ Th2 cells and its production of IL-4 and IL-10 that have protective effects. IL-2 and IFNγ also can activate both macrophages to synthesize β-cell toxic IL-1, TNFα, IFNγ, NO; and β-cell cytotoxic $CD8^+$ cells with the additional production of IFNγ. GCs can suppress the immunoresponse by inactivating macrophages, $CD4^+$ Th1, $CD8^+$cells, along with their associated cytokines and activate $CD4^+$ Th2 T regulatory cells.

Basal plasma corticosterone level is significantly increased in STZ induced diabetic rats (Chan et al. 2005), with no change in GR content in the pancreas (Svec 1985; Ranhotra and Sharma 2000). However, whether GCs impacts upon STZ induced diabetes is unknown.

11β-HSD1 is the enzyme that amplifies glucocorticoid action in many tissues (Described in Chapter 1). 11β-HSD1 is predicted to have an anti-inflammatory role as it is expressed in immune cells and regulates both innate and adaptive immune responses (reviewed in Chapman et al. 2009). 11β-HSD1 is expressed in pancreatic islets but so far no one reported an anti-inflammatory effect of 11β-HSD1 in this tissue.

5.2 Hypothesis

Our initial hypothesis was that elevated local GC regeneration would have a negative effect on GSIS, thus promoting diabetes in a model of diet-induced β-cell failure. However, having shown that chronically but modestly elevated β-cell 11β-HSD1 rescued β-cell function, we went on to hypothesise that:

Elevated β-cell 11β-HSD1 protects β cells from STZ induced β-cell damage.

5.3 Aims

To test the effects of β-cell-specific overexpression of 11β-HSD1 on:

1. β-cell destruction of STZ-induced diabetes
2. β-cell regeneration after STZ treatment
3. Inflammatory responses to STZ treatment
4. β-cell apoptosis after STZ treatment

5.4 Experimental Design

5.4.1 Induction of type 1 diabetes in mice with streptozotocin

Low-dose STZ: Mice were treated i.p. for 5 consecutive days with saline or 40mg/kg/BW STZ dissolved in saline. Blood samples were taken from the tail vein at day 0, 4, 12, and 15 for glucose and insulin levels.

High-dose STZ: Mice were given a single bolus i.p. injection of either 10mM citrate buffer (pH4.5) or STZ (180mg/kg/BW, dissolved in 10mM citrate buffer). Blood glucose was measured daily via tail venesection. Mice were sacrificed at 3 and 10 days after STZ treated.

Experimental mice were age matched (10-12 week). Blood samples were taken at day one, and trunk blood was collected on the last day. Heads of pancreata were harvested and frozon at -80°C and tails of pancreata were fixed in 10% formalin for IHC.

5.4.2 Assessment of diabetes after streptozotocin treatment

Plasma glucose was measured by glucose meter (Described in chapter 2.6.1.), and insulin level was measured by mouse insulin ELISA kit (Described in chapter 2.6.2.). Morphology and insulin content was assessed by IHC and H&E counterstain (Described in chapter 2.16)

5.4.3 Assessment of β-cell proliferation after streptozotocin treatment

Ki67 and PDX1 were co-stained by IF (Described in chapter 2.17). Ki67 is a nuclear protein expressed in proliferating cells and is present during all active phases of the cell cycle, but is absent from resting cells (G_0). Ki67 is used as a cellular marker for

proliferation (Bullwinkel et al. 2006). Pancreatic and duodenal homeobox1 (PDX1) is an important insulin gene transcription factor, and a necessary transcriptional factor for pancreatic development and β-cell maturation (Hui and Perfetti 2002). Cells co-expressing Ki67 and PDX1 thus delineate proliferation occurring in insulin producing cells which was interpreted as β-cells undergoing proliferation or replication. Quantitation for PDX1 and Ki67 positive cells was as described in chapter 2.2.12.

5.4.4 Assessing the inflammatory status of the pancreas after streptozotocin treatment

Mac-2 also known as Galectin-3 (Gal-3), eBP, IgE binding protein, CBP-35, CBP30, L-34 and L-29 is a 32-kDa β-galactosidase-binding lectin widely distributed in cells, but is enriched in cells of the monocyte/macrophage lineage 6. Mac-2 is widely used as an activated macrophage marker (Liu et al. 1995; Dumic et al. 2006) to detect macrophage infiltration.

Foxp3 (X-linked forkhead/winged helix transcription factor) is expressed at the highest level in $CD4^{+}CD25^{+}$ T regulatory cells and has emerged as an important marker of T regulatory cells (Fontenot and Rudensky 2005). Quantitation was carried out for Foxp3 (Described in chapter 2.2.12).

5.4.5 Assessment of β-cell neogenensis after streptozotocin treatment

Pancreatic β-cell neogenesis was determined with Neurogenin 3 (Ngn3) and SRY/HMG box 9 (Sox9) within pancreatic tissue. Neurogenin 3 (Ngn3) is a basic helix-loop-helix protein which is detected in a currently ill-defined subpopulation of cells scattered within the early pancreatic region during development, and is required for endocrine cell development (Gradwohl et al. 2000). Ngn3 is widely used to detect newborn endocrine cells in injured pancreas. Sox9 is expressed in the adult pancreatic ductal epithelium (Akiyama et al. 2005) and is essential for pancreatic

development and activation of the endocrine cell differentiation program (Lynn et al. 2007).

5.4.6 Detection of β-cell apoptosis after streptozotocin treatment

Apoptotic pancreatic β-cells were detected by deoxynucleotide transferase (TdT) mediated dUTP nick-end labeling (TUNEL) assay. The TUNEL system measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP at 3'-OH DNA ends using the enzyme terminal TdT, which forms a polymeric tail using the principle of the TUNEL assay. TUNEL was widely used to detect apoptotic β-cells in NOD mice (Kurrer et al. 1997; Augstein et al. 1998; Augstein et al. 1998; Kim et al. 1999).

Caspase-3 is a critical mediator of apoptosis, being responsible for the proteolytic cleavage of many key proteins in this process (Fernandes-Alnemri et al. 1994). An antibody to cleaved caspase-3 was used to detect pancreatic apoptosis in whole pancreas lysates (Aizman et al. 2010).

5.5 Results

5.5.1 MIP-HSD1$^{tg/+}$ mice show transiently reduced hyperglycaemia diabetes with high-dose STZ

5.5.1.1 MIP-HSD1$^{tg/+}$ mice show partial rescue of hyperglycaemia induced by high-dose of STZ

High-dose STZ (180mg/kg/BW) causes massive β-cell destruction and leads to permanent and profound hyperglycaemia. All genotypes became hyperglycaemic within two days of high dose STZ and there was no significant difference among the groups suggesting equal severity of the initial insult and β cell destruction. However, after two days MIP-HSD1$^{tg/+}$ mice exhibited a transient, small but significant oscillatory improvement in their hyperglycaemia. (Figure 5-4)

Three days after high-dose STZ treatment, plasma insulin level was markedly decreased in KsJ, MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ mice (Figure 5-5). However, MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ mice maintained higher insulin levels than KsJ at day 3, consistent with their oscillatory protection from hyperglycaemia. By day 10, MIP-HSD1$^{tg/+}$ maintained a higher insulin level than KsJ.

Pancreatic tissue was stained for insulin protein by immunohistochemistry. In all genotypes of mice, insulin was localized specifically within the islets. Islet number and size was reduced at day 3 and 10 post STZ, most noticeably in KsJ mice compared to MIP-HSD1 mice which clearly exhibited stronger insulin staining consistent with higher plasma insulin levels (Figure 5-6).

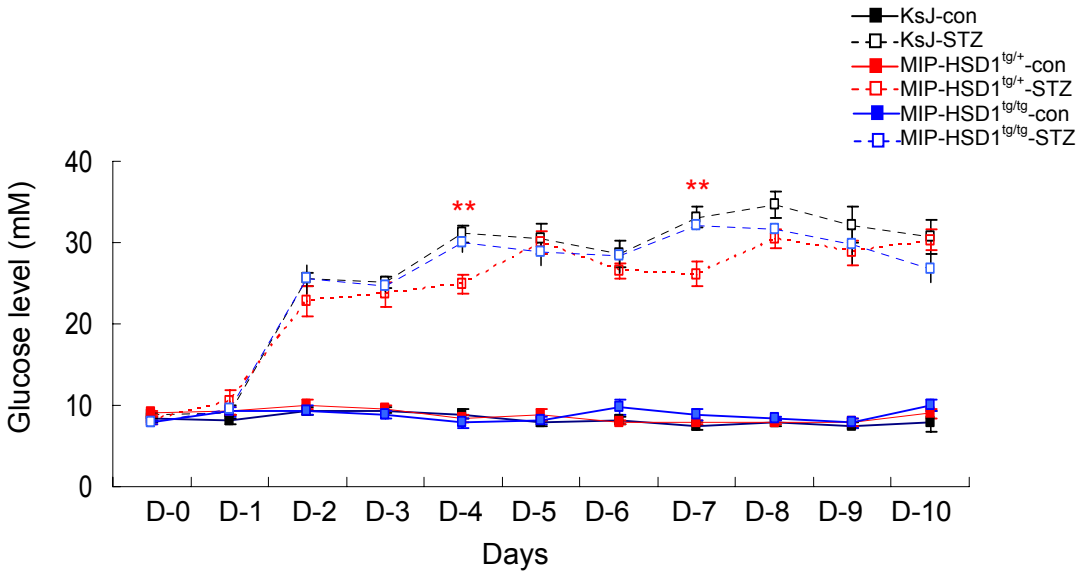


Figure 5-4. MIP-HSD1$^{tg/+}$ mice exhibit a transient, oscillatory improvement in hyperglycaemia after high dose STZ. Age-matched mice were given a single bolus i.p. injection of either 10mM citrate buffer (pH4.5) or STZ (180mg/kg/BW, dissolved in 10mM citrate buffer). Blood glucose was measured via tail vein venesection everyday for 10 days. Values represent the mean ± S.E.M, **P<0.01, (One-way ANOVA, Tukey's Multiple Comparison Test), control group n=5-7, STZ experiment group n=6-10.

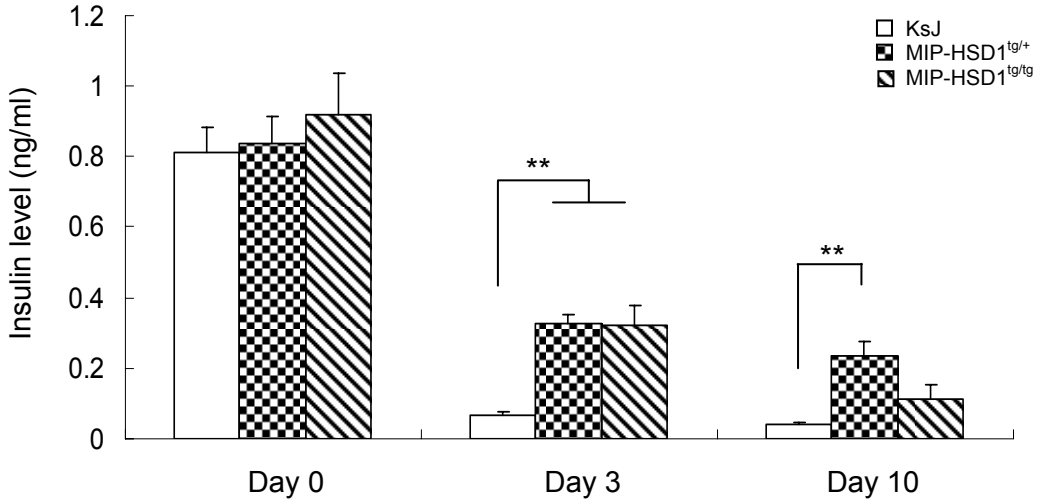


Figure 5-5. MIP-HSD1 mice maintain a higher insulin level than KsJ after high dose STZ. Age-matched mice were given a single bolus i.p. injection of either 10mM citrate buffer (pH4.5) or STZ (180mg/kg/BW, dissolved in 10mM citrate buffer). Plasma insulin was measured in blood samples the day before STZ injection and at day 3 and day 10 post STZ injections. Values represented the mean ± S.E.M, **P<0.01 (One-way ANOVA, Tukey's Multiple Comparison Test), control group n=5-7, STZ experiment group n=6-12.

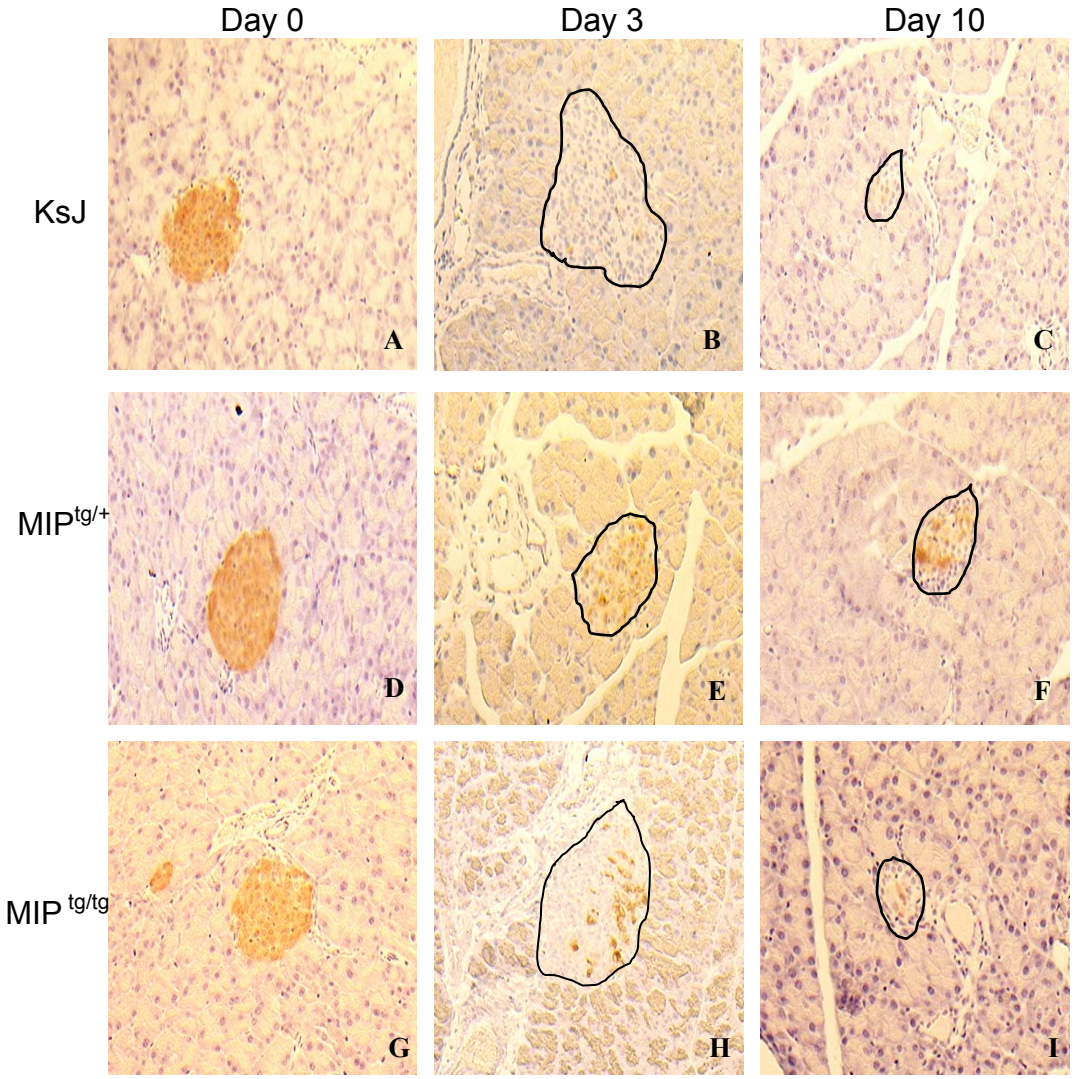


Figure 5-6. MIP-HSD1$^{tg/+}$ islets exhibit higher insulin content after high dose STZ. Pancreas was fixed in 10% formalin and embedded in paraffin/wax. Immunohistochemistry staining for insulin was achieved using guinea pig anti-insulin (1:300 dilution) performed on 4µm section. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Islet capsule is indicated by the black line around its perimeter. Magnification ×10.

5.5.1.2 MIP-HSD1$^{tg/+}$ mice maintain higher β-cell mass and proliferation after high-dose STZ

To explore how the MIP-HSD1$^{tg/+}$ could partially resist high-dose STZ induced hyperglycaemia, we investigated their β-cell proliferation capacity using immunofluorenscence by co-staining with Ki67 and PDX1 (Described in 5.3.4).

Control pancreas exhibited proliferating β-cells in both KsJ and MIP-HSD1 transgenic mice (Figure 5-7). After high dose-STZ injection, β-cell number reduced dramatically in all groups. We were unable to observe PDX1 staining at day 10 in KsJ mice. MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ had higher numbers of remaining β-cells (insulin) after 3 days and clearly detectable β-cells at day 10 (Figure 5-7).

β-cell proliferation was comparable between KsJ and MIP-HSD1 mice in the vehicle-treated group (Figure 5-8). At day 3 post STZ, PDX1 staining and therefore β-cell number decreased in all groups, with the greatest decrease observed in KsJ mice. Ki67^{+} proliferating cells decreased from day 3 to day 10 in KsJ mice, as did PDX1 staining, which was absent in pancreas of KsJ mice at day 10. MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ mice maintained PDX1^{+} cells (β-cell) and higher Ki67^{+} cell numbers at day 10 than KsJ mice. MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ mice had detectable PDX1/Ki67 double-positive cells (proliferating β-cell) at day 10 (Figure 5-8). Overall this suggests MIP-HSD1$^{tg/+}$ and MIP-HSD1$^{tg/tg}$ mice retain a higher capacity for replicative replacement of functional β-cell after high dose STZ.

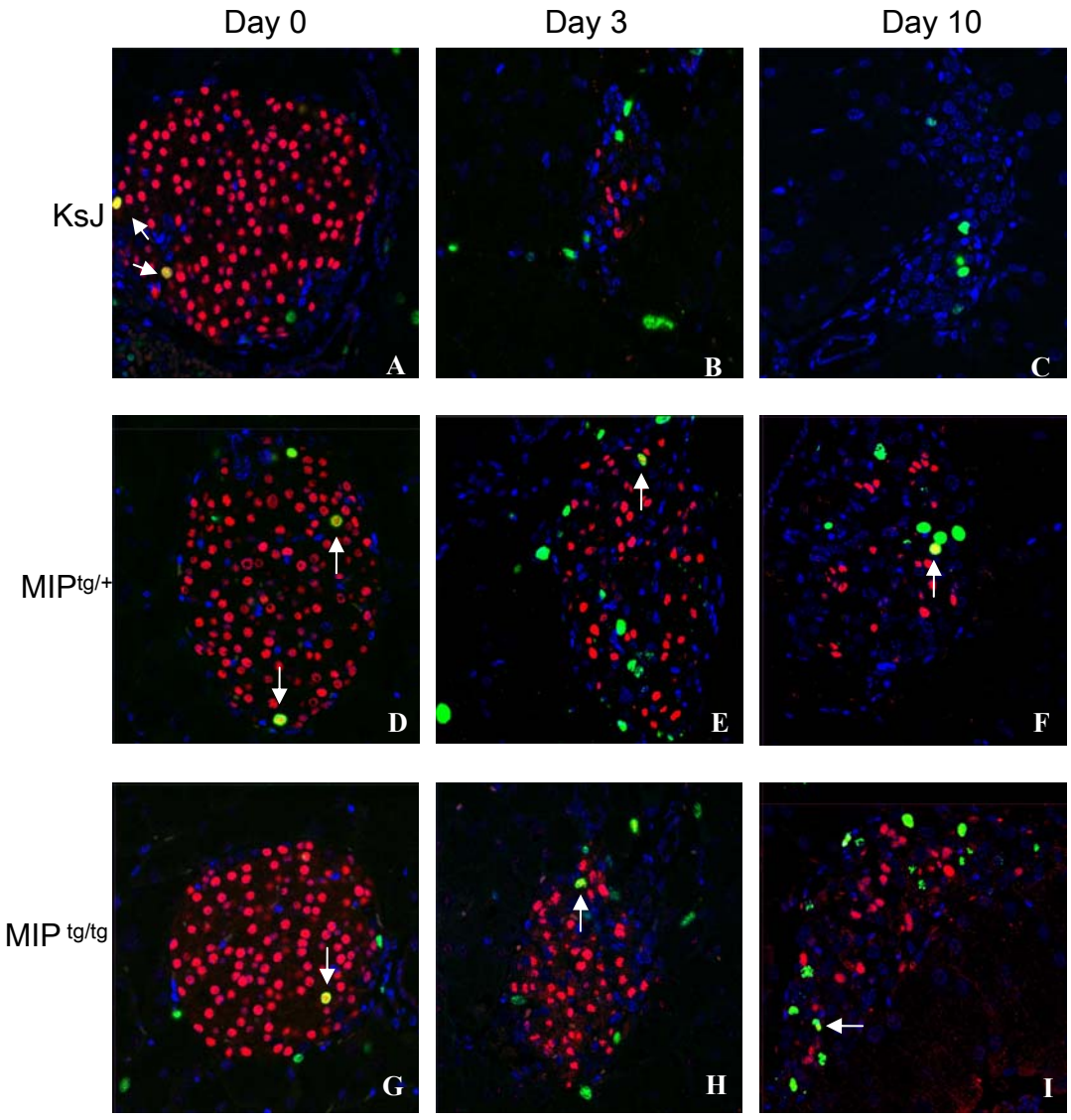


Figure 5-7. MIP-HSD1 mice have a higher β-cell proliferative cell number after STZ treatment. Pancreas sections were stained using immunofluorenscence co-stained by rabbit anti-Ki67 (1:3000 dilution, green), rabbit anti-PDX1 (1:1000 dilution, red) and, DAPI (1:1000 dilution, blue) as a nuclear stain. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Arrows indicate double positive stained cells, magnification ×40.

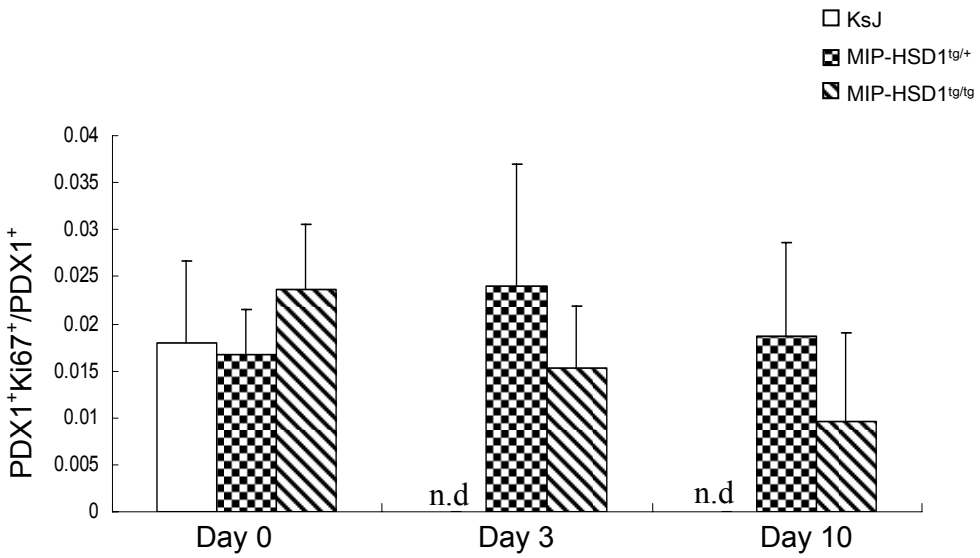


Figure 5-8. Higher double PDX-1 and Ki67 positive staining cells in islets of MIP-HSD1 mice. Quantitation using Image J software was employed for the light microscopy images. PDX1 and Ki67 positive cells were counted within the islets as defined morphologically by the islet capsule boundary through the section. The rate of proliferating β-cells was presented as ratio of PDX1, Ki67 double-positive cells to PDX1 positive cells in each islet then averaged for each group. PDX1 was undetectable for KsJ STZ treated for 3 and 10 days (n.d). Values represented the mean ± S.E.M, (One-way ANOVA, Tukey's Multiple Comparison Test), control group n=5-7, STZ experiment group n=6-12.

5.5.1.3 MIP-HSD1$^{tg/+}$ mice show ameliorated islet inflammation after high-dose STZ

5.5.1.3.1 MIP-HSD1$^{tg/+}$ mice showed reduced macrophage infiltration after high-dose STZ

Islet macrophage infiltration is an early event where inflammation is initiated in T1D and STZ-induced diabetes. Critically, macrophage infiltration is an absolute requirement for the development of T1D in animal models (Mandrup-Poulsen 1996) as potent and critical immune regulators (Kolb et al. 1990; Lukic et al. 1998; Homo-Delarche and Drexhage 2004). Locally elevating the GCs level can suppress inflammation by inhibiting lymphocyte proliferation and cytokine production. As a consequence, tissue macrophage responses are also affected (Gillis et al. 1979; Larsson 1980). To investigate whether MIP-HSD1 protected β-cells from anti-inflammatory effects, pancreatic tissue was stained with Mac-2 antibody which is a marker for activated macrophages (Described in 5.2.1).

Before STZ injection, KsJ and MIP-HSD1 had very few macrophages within the islets and among the acinar tissue. On day 3 after STZ, there was an increase in the number of activated macrophages in KsJ islets compared to MIP-HSD1. The active macrophage numbers were diminished by day 10 in all genotypes (Figure 5-9).

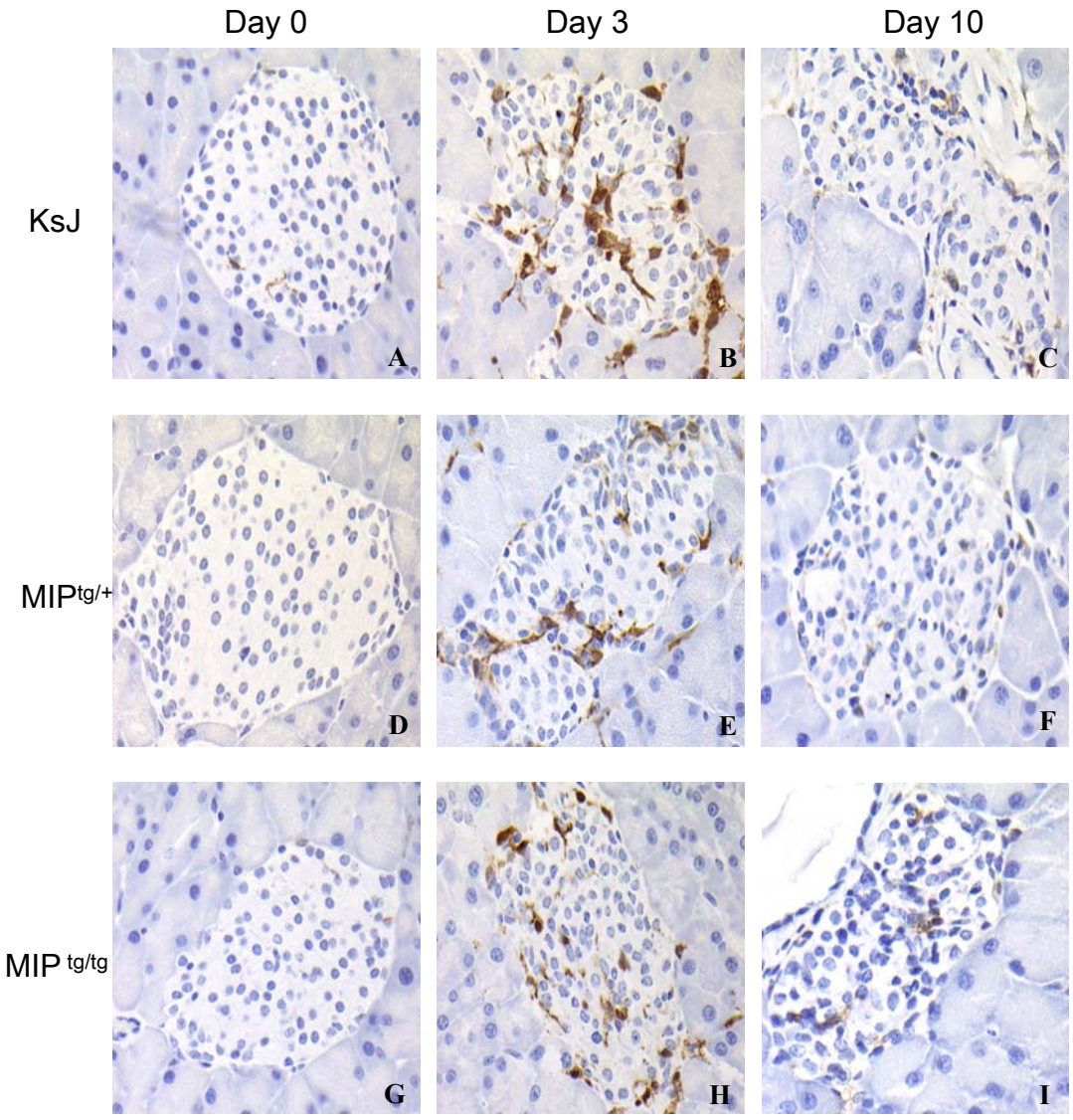


Figure 5-9. MIP-HSD1 mice exhibit reduced islet macrophage infiltration at day 3 after STZ treatment. Pancreas was fixed in 10% formalin and embedded in paraffin/wax. Immunohistochemistry staining for Mac-2 using rabbit anti-mac-2 (1:150 dilution) performed on pancreas sections. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Magnification ×40.

5.5.1.3.2 MIP-HSD1$^{tg/+}$ mice exhibit a pronounced Foxp3 T-regulatory cell infiltration after high-dose STZ

T regulatory cells are a subpopulation of T cells that maintain immune system homeostasis by suppressing immune responses (Van Parijs and Abbas 1998). To test whether MIP-HSD1 showed evidence of immunosuppressive T regulatory cells, we stained with the Treg marker Foxp3 (Described in 5.2.2).

Very few T regulatory cells were found in normal pancreas of all groups under normal conditions. Three days post STZ treatment, T regulatory cells were slightly increased in all genotypes, with a pronounced pattern within or surrounding the remaining islets. However, 10 days post STZ, MIP-HSD1$^{tg/+}$ mice showed evidence for markedly increased T regulatory cell number relative to KsJ and MIP-HSD1$^{tg/tg}$ mice (Figure 5-10).

The number of Foxp3 positive cells was quantified in all groups of mice by computerized image analysis. T regulatory cell content was presented as the ratio of Foxp3^{+} cells relative to the area of whole pancreas. Notably, in non-STZ treated pancreas, MIP-HSD1$^{tg/tg}$ mice showed a significantly higher T regulatory cell content versus KsJ. Three days post STZ treatment, all genotypes tended to increase T regulatory cell content but not significantly. Ten days post STZ treatment, T regulatory cells clearly increased in all groups compared to day 0. The greatest increase (10-fold) in T regulatory cell content was observed in MIP-HSD1$^{tg/+}$ mice (Figure 5-11).

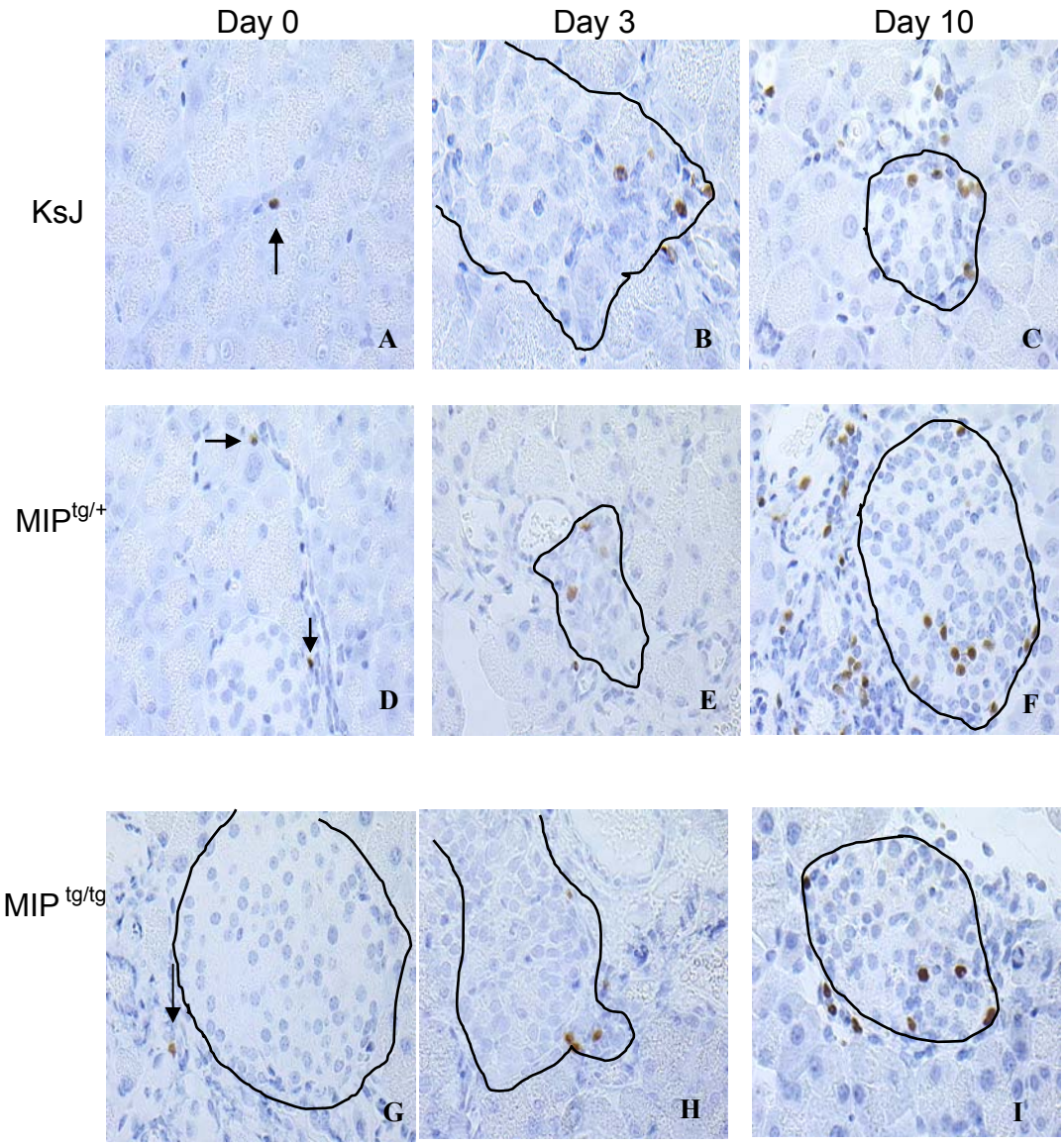


Figure 5-10. MIP-HSD1$^{tg/+}$ mice exhibit markedly increased T-regulatory cell numbers ten days after STZ administration. Pancreas was fixed in 10% formalin and embedded by paraffin/wax. Immunohistochemistry staining for Foxp3 using rat anti-Foxp3 (1:150 dilution) was performed on pancreas section. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Islet capsule is indicated by the black line around its perimeter. Magnification ×40

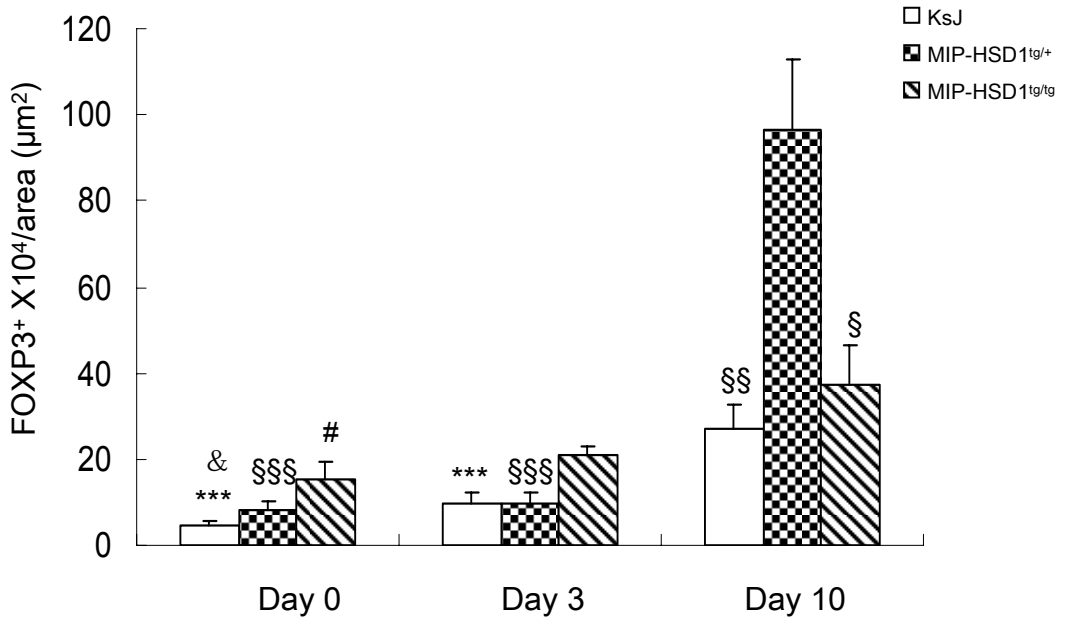


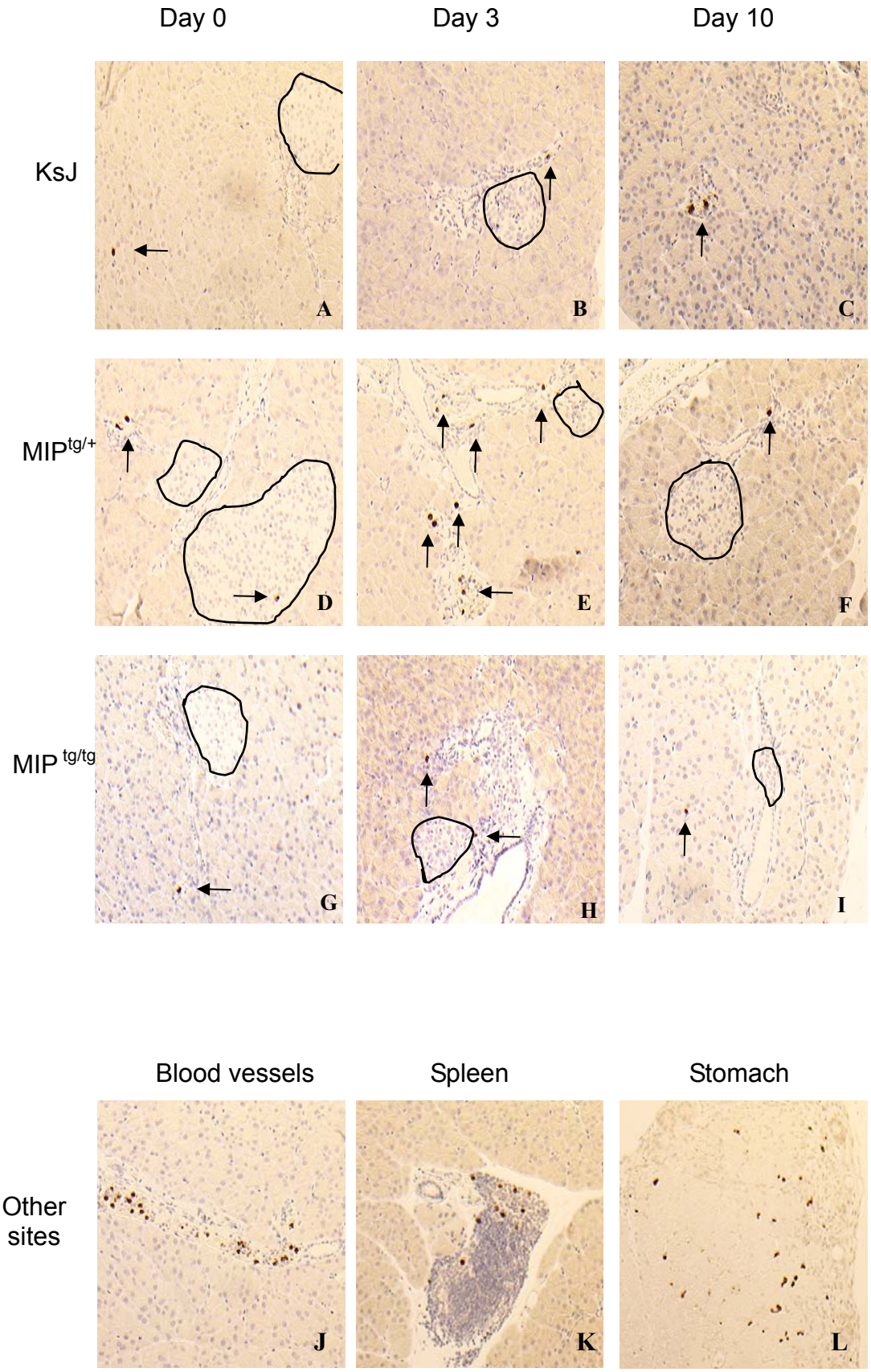
Figure 5-11. Quantitation of T regulatory cells in MIP-HSD1 pancreas with and without STZ. Quantitation using computerized image analysis. Foxp3 positive cells were counted by Zeiss (KS300, 3.0) software throughout the section; section area was measured by MCID Basic 7.0. Each individual mouse value was calculated from averaging at least three sections, which were more than 100µm apart (to avoid counting the same islet twice). Y-axis represents the ratio of positive Foxp3 cells to whole section area. Values represent mean ± S.E.M, ***P<0.0001, (vs. KsJ Day 10), §§§P<0.0001, §§P<0.01, §P<0.05 (vs. MIP-HSD1$^{tg/+}$ Day 10), # P<0.05 (vs. MIP-HSD1$^{tg/tg}$ Day 10), & P<0.05 (vs. MIP-HSD1$^{tg/tg}$ Day 0) (One-way ANOVA, Tukey's Multiple Comparison Test), control group n=5-7, STZ experiment group n=6-10.

5.5.1.4 Investigation of islet neogenesis with Neurogenin 3 and Sox 9 after high-dose STZ

β-cell neogenesis is considered as an important mechanism counteracting hyperglycaemia induced by β-cell injury. Among the progenitor markers, Neurogenin 3 (Ngn3) which is important for endocrine cells during pancreatic development has been widely used to detect β-cell neogenesis. In addition, Sox9 is a marker recently found to be essential for pancreatic development and the regulation of endocrine cell differentiation (Akiyama et al. 2005).

Figure 5-12 shows that Ngn3 positive cells are scattered throughout pancreatic tissue, a few of them within the islets. Groups of Ngn3 positive cells are found in blood vessels, and are highly prevalent in spleen and stomach. Sox9 is expressed in adult pancreatic ductal epithelium. Therefore, the Sox9 positive cells were widely spread in pancreas (Figure 5-13). The computer imaging analysis method (Image Pro MC software) was used to quantify Sox9 positive cells by comparing the density of Sox9 positive area on 13 to 30 random selected areas per section per mouse. There was no difference observed between day 0 and day 3 after STZ in KsJ mice. However MIP-HSD1$^{tg/+}$ mice showed significantly a reduced Sox9 expression. In contrast, Sox9 expression was increased in MIP-HSD1$^{tg/tg}$ after STZ treatment for 3 days. (Figure 5-14)

Figure 5-12. Ngn3 staining is scattered in adult pancreas compared to other tissues. Pancreas was fixed in 10% formalin and embedded by paraffin/wax. Immunohistochemistry staining for Ngn3 using rabbit anti-Ngn3 (1:1000 dilution) performed on pancreas section. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Ngn3 positive cells were scattered in pancreatic tissue (A-I) or group appeared in blood vessels (J), or expressed in spleen (K) and stomach (L). Islet capsule is indicated by the black line around its perimeter. Magnification ×10


Day 0
Day 3
Day 10
KsJ
MIP tg/+
MIP tg/tg
A
B
C
D
E
F
G
H
I
Blood vessels
Spleen
Stomach
Other sites
J
K
L

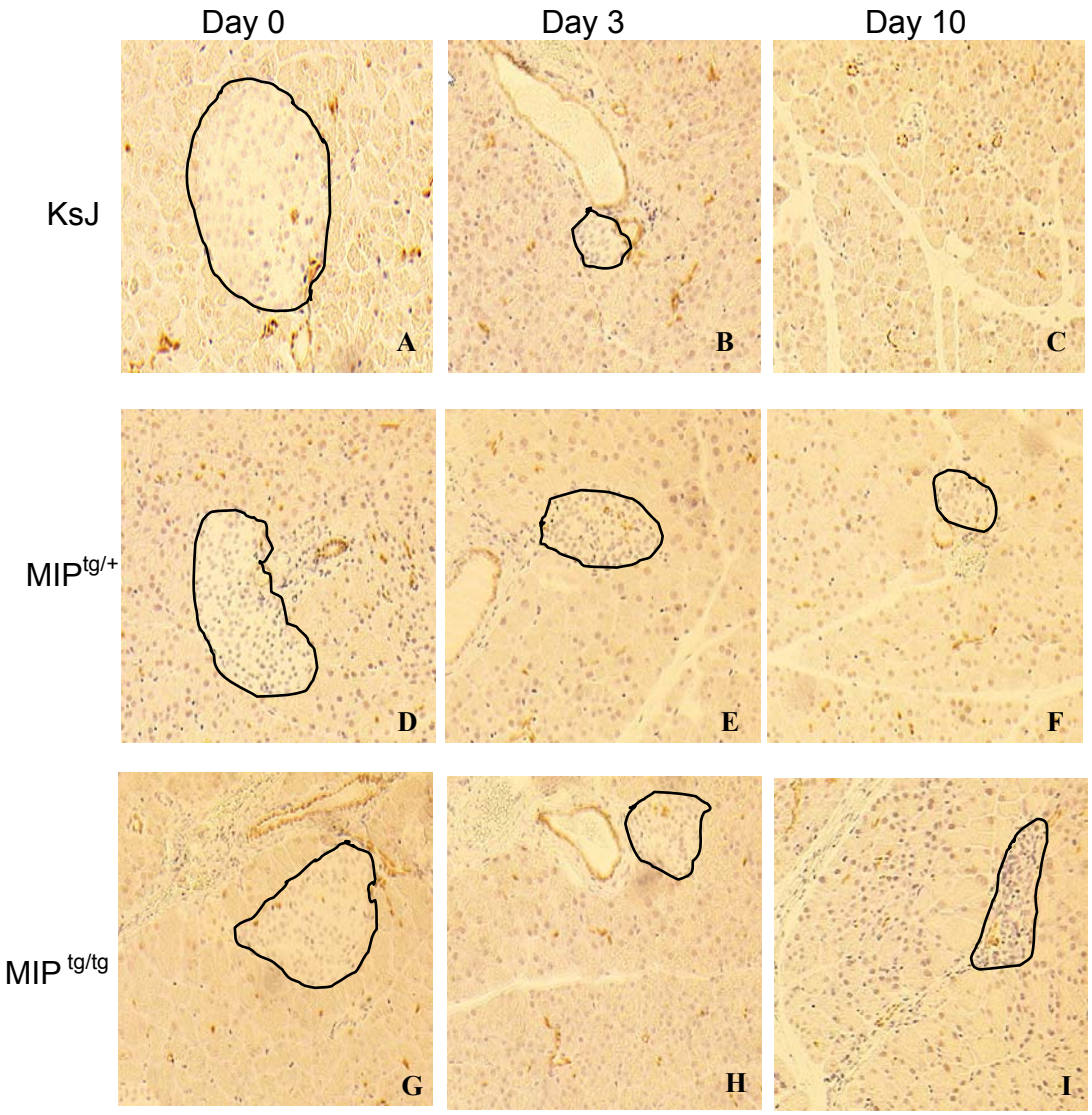


Figure 5-13. Sox9 is expressed in ductal cells and widely scattered in undefined cells in the pancreas. Pancreas was fixed in 10% formalin and then embedded in paraffin/wax. Immunohistochemistry staining for Sox9 was with a rabbit anti-Sox9 (1:8000 dilution) anti-body and was performed on pancreas sections. (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treated, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Islet capsule is indicated by the black line around its perimeter. Magnification ×10

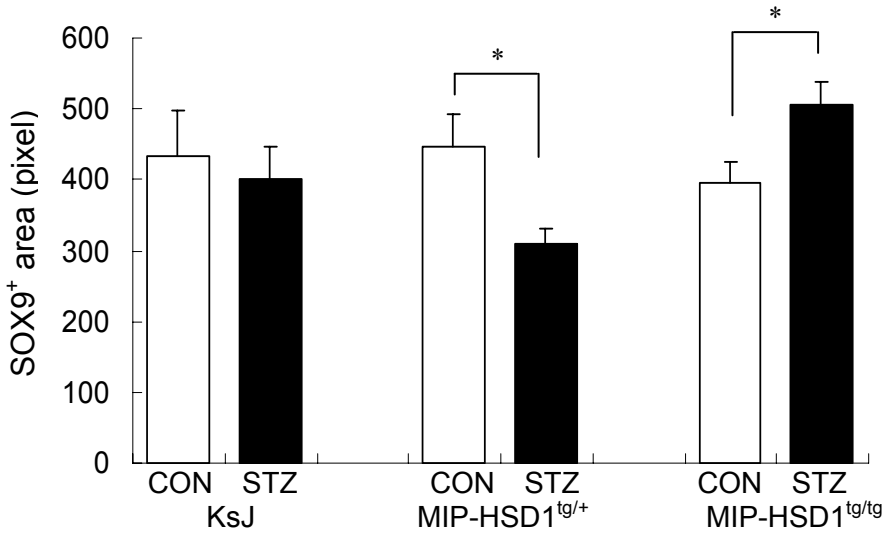


Figure 5-14. Quantitation of Sox9 in the pancreas after 3 days of STZ treatment. Pancreas was fixed in 10% formalin and embedded in paraffin/wax. Immunohistochemistry staining for Sox9 was obtained using rabbit anti-Sox9 (1:8000 dilution) performed on pancreas section. Quantitation was achieved using computerized image analysis. Sox9 positive cells area density was counted by Image Pro MC software throughout the section. Values represent mean ± S.E.M, * $P<0.05$ (One-way ANOVA, Tukey's Multiple Comparison Test), control group n=5-7, STZ experiment group n=6-10.

5.5.1.5 Investigation of islet apoptosis after high-dose STZ

TUNEL assay was used to detect β-cell apoptosis. Almost no apoptotic cells were found in the pancreatic tissues regarding the genotypes and treatments. In addition Western blot was used to determine the level of cleaved caspase-3 which gives an indication of the activation of the apoptotic pathway (apoptotic cells marker). Pancreatic lysates of KsJ mice at different days after STZ were blotted with cleaved caspase-3 antibody. Spleen was used as a positive control. Consistent with TUNEL results there was no specific apoptotic bands in KsJ mouse pancreas, while the positive control spleen had a specific band (Figure 5-15). Therefore, using whole pancreas resulted in a loss of sensitivity to detect apoptosis within islets by this method. Apoptosis levels were clearly low in the high dose STZ model and the lack of quantifiable cell numbers made it impossible to draw any conclusions on the impact of MIP-HSD1 on apoptosis in islets. The tentative conclusion is that there is no significant effect on this process in this model.

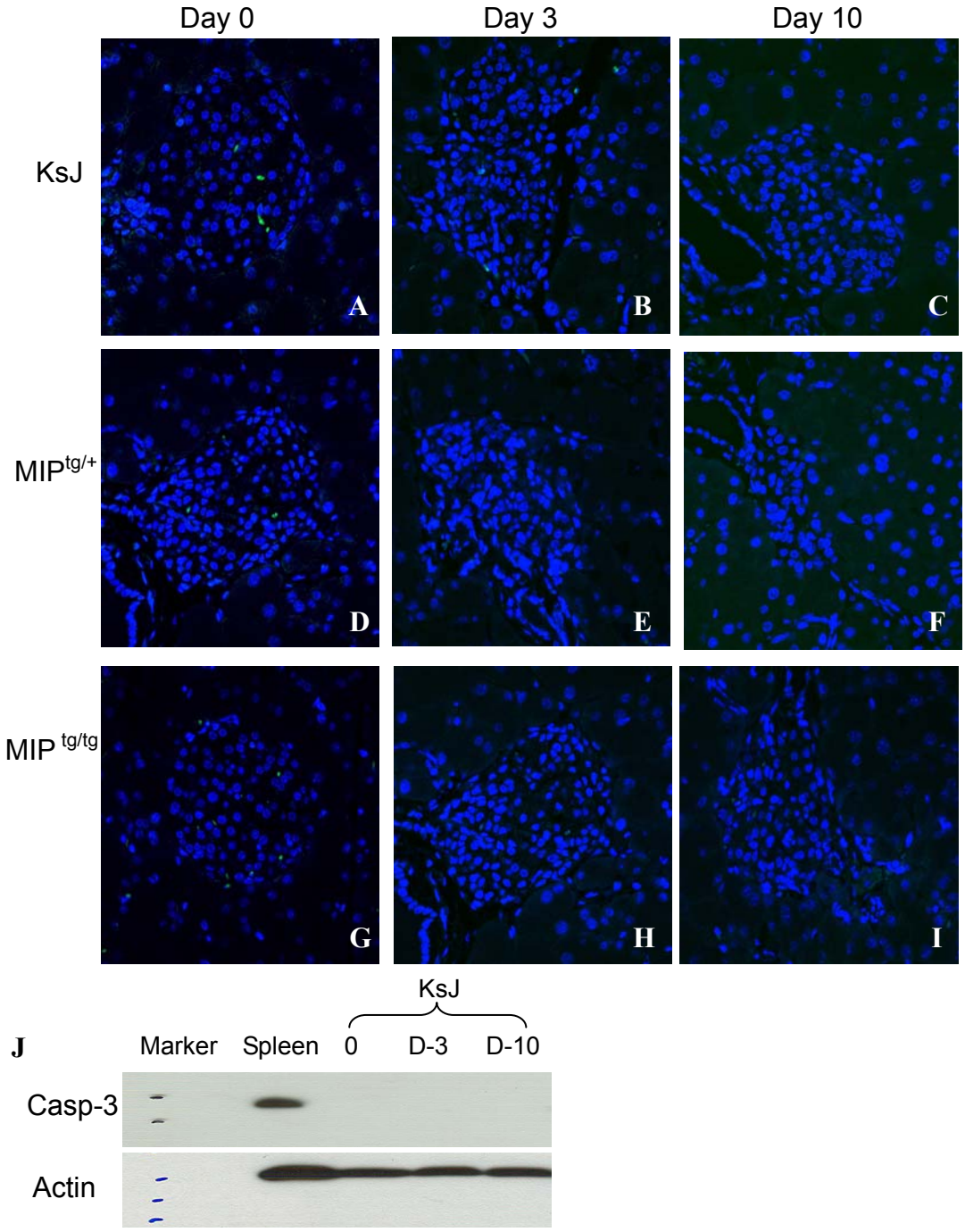


Figure 5-15. Evaluation of β-cell apoptosis after STZ treatment. TUNEL staining for paraffin fixed pancreas. Apoptotic cells were stained by TUNEL (green), nuclei were stained with DAPI (blue). No difference was found between the groups. Representative pictures shown above were (A-C) KsJ, (D-F) MIP-HSD1$^{tg/+}$, (G-I) MIP-HSD1$^{tg/tg}$; (A, D, G) without STZ treatment, (B, E, H) STZ treated for 3 days, (C, F, I) STZ treated for 10 days. Magnification ×40. Cleaved Caspase-3 (Casp-3, 1 in 1000 dilution, 17kDa) was performed in KsJ pancreas by western blot. Spleen was used as a positive control, actin was used as internal loading control. No bands were observed for caspase-3 in KsJ whole pancreas tissue with or without STZ treatment. A representative image is shown (J).

5.5.2 MIP-HSD1$^{tg/+}$ mice resist multiple low-dose STZ-induced mild hyperglycaemia

Low-dose STZ causes delayed onset mild diabetes through β-cell damage and immunological injury. KsJ and MIP-HSD1$^{tg/+}$ mice were treated with low-dose STZ (40mg/kg/BW) for five days. In the STZ-treated KsJ group, glycaemia increased from day 4. After day 12, the KsJ group became maximally hyperglycaemic for this dose of STZ. However, the increase of plasma glucose levels in the MIP-HSD1$^{tg/+}$ group was clearly attenuated compared with KsJ at day 4 and 12. (Figure 5-16)

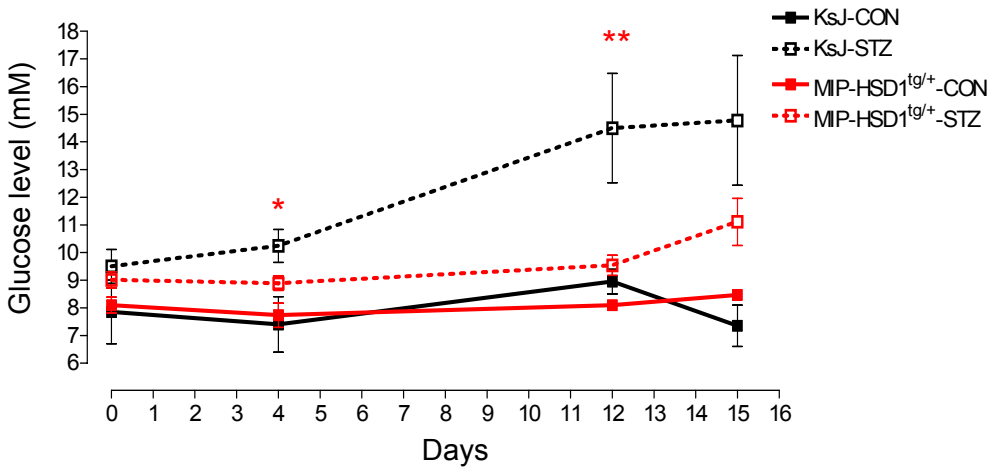


Figure 5-16. Plasma glucose level after multiple low-dose STZ. Mice were i.p. injected either with saline or STZ (40mg/kg/BW) dissolved in saline for 5 consecutive days. Blood samples were taken from tail at day 0, 4, 12, and 15. Black solid line-KsJ control group; red solid line-MIP-HSD1$^{tg/+}$ control group; black dots line-KsJ STZ group; red dots line-MIP-HSD1$^{tg/+}$ STZ group. Values represented the mean ± S.E.M, **P<0.005, *P<0.05, (Student t-test), control group n=3, STZ experiment group n=5.

5.6 Discussion

The present study demonstrated that chronic modest elevation 11β-HSD1 specifically in pancreatic β-cells caused a small but significant reduction of the hyperglycaemia induced by high-dose and low-dose STZ treatment.

The protection from diabetes was mediated by an increased number of remaining β-cells. Since the initial insult with high dose STZ led to similar hyperglycaemia, it suggests either a qualitatitive difference in the initial insult followed by an unmasking or improved survival of the existing MIP-HSD1 β-cells, or that the recovery of β-cell through other means is improved in MIP-HSD1 mice, or both. Methodological limitations made it impossible to formally measure any increase in β-cell neogenesis with the currently advocated markers Ngn3 (successfully used in a pancreatectomy model) and Sox9. There were greater numbers of PDX^{+}-$Ki67^{+}$ cells indicative of maintained β-cell proliferation in the MIP-HSD1 islets. Moreover, there was clear evidence for reduced pro-inflammatory macrophage infiltration and increased immuno-regulatory, pro-resolution mediating $Foxp3^{+}$ T regulatory cells.

The loss of β-cells was more rapid and severe in KsJ mice after STZ, whereas the number of proliferating cells (non-PDX^{+}) which surrounded the destroyed islet region increased. These non-endocrine proliferating cells may consist of inflammatory cells and/or α-cells, which are increased in STZ-treated mice (Li et al. 2000).

Macrophage infiltration is an early response to the islet release of pro-inflammatory cytokines, such as IL-1, IFNγ and TNFα which are important for β-cell destruction (Mandrup-Poulsen 1996). Moreover, depletion or inactivation of macrophages prevents the development of T1D (Oschilewski et al. 1985; Hutchings et al. 1990). MIP-$HSD1^{tg/+}$ mice had reduced macrophage infiltration three days after high-dose STZ. This is likely due to decreased release of pro-inflammatory cytokines resulting

in a lower severity β-cell destruction and death. Ten days after high-dose STZ, the phenomenon of macrophage infiltration was reduced in all genotypes suggesting this pro-inflammatory response to dying β-cells and their clearance was complete. Notably, the Mac-2 (galectin-3) is expressed on macrophages and monocytes and it regulates immunoregulatory function of macrophages. Deletion of galectin-3 attenuated multiple low-dose STZ induced hyperglycaemia (Mensah-Brown et al. 2006), further emphasising its expression here as a read out of the inflammatory macrophage response.

T lymphocytes (T-cells) are also widely accepted as important effectors in T1D. It has been hypothesized that the pathogenic cellular immune response is mediated by Th1 cells, a subset of T cells characterized by secretion of IL-2 and INFγ which induce macrophages and $CD8^+$ cells to secrete proinflammatory cytokines. Alternately, the protective humoral immune response is mediated by Th2, a subset of T cells that synthesize IL-4 and IL-10 which are considered as anti-inflammtory effectors (Rabinovitch 1994). The balance between Th1 and Th2 within the islet microenvironment may influence the development of T1D. $CD4^+CD25^+$ (Th2) cells known as T regulatory cells (Treg) constitute 5-10% of peripheral $CD4^+$ T cells (Sakaguchi 2004). Although the nature and diversity of Treg cells remains unclear, Foxp3 which is expressed at the highest level in $CD4^+CD25^+$ T cells has emerged as an important marker for Tregs (Fontenot and Rudensky 2005). The MIP-$HSD1^{tg/+}$ mice exhibited significantly increased Treg cell numbers after ten days high-dose STZ treatment. This could reflect the modestly higher level of active GCs which have known modulatory effects on induction of a Th2 response (Ramirez 1998). Furthermore, the high level of Tregs in MIP-$HSD1^{tg/tg}$ mice even in the absence of STZ treatment suggests that a higher level of 11β-HSD1 may affect a subtle change within the islet microenvironment leading to an effectively initial resistance to the inflammatory insult. This is consistent with a later dimunition of Tregs in MIP-$HSD1^{tg/tg}$ compared to MIP-$HSD1^{tg/+}$ at ten days post-injection. However, three days after STZ, there was no change in Treg numbers in any genotypes, consistent with an early pro-inflammatory response followed by a gradual recruitment and replacement with resolving, immune-dampening Tregs.

Although neogenesis of β-cells in adult pancreas is still controversial (Dor et al. 2004; Teta et al. 2007), β-cell regeneration from a variety of progenitor cells occurs in several pancreatic injury models (Fernandes et al. 1997; Zulewski et al. 2001; Hayashi et al. 2003; Xu et al. 2008; Sangiorgi and Capecchi 2009). This is a potential mechanism whereby the pancreas regenerates insulin producing cells from other cell types, and therefore could mediate diabetes-resistance. However, there is currently no satisfactory and specific marker to detect neogenesis of β-cells in adult injury response to STZ. The marker widely used is Neurogenin 3 (Ngn3) which is important for endocrine cell differentiation during neonatal pancreatic development. Sox9 was also recently found to be essential for pancreatic development and endocrine cell differentiation: pancreatic cells from all lineages derive from Sox9-expressing precursors (Akiyama et al. 2005). Ngn3 positive cells were scattered in pancreatic tissue, with prominent staining in blood vessels in the pancreas, and high expression in spleen and stomach. It is concluded that this marker is not specific for pancreatic endocrine precursor cell staining in the adult STZ-injury mouse model.

Sox9 changes suggested that progenitor cells are differentiated more extensively to endocrine cells in MIP-HSD1$^{tg/tg}$ after 3 days STZ treatment. However, this data must be interpreted with caution as the MIP-HSD1$^{tg/+}$ mice that exhibit a marked protection from diabetes indeed greater than MIP-HSD1$^{tg/tg}$ in response to high-dose STZ, showed lower Sox9 expression 3 days after STZ treatment. Sox9 is expressed in adult pancreatic ductal epithelium but also in ill-defined cells throughout the pancreas. Therefore, the Sox9 staining requires careful further co-localisation with β-cell specific markers to determine cell type specific expression patterns. It is intriguing that the Sox9 expression shows a U-shaped dose-response opposite to that of the MAC2 staining pattern. This suggests an impact of the transgene induced GC levels, although the exact meaning of this change is unclear.

Apoptosis is a major form of β-cell death in rodent T1D models, as high glucose contributes to β-cell apoptosis (Ohneda et al. 1995; Donath et al. 1999; Liu et al.

2000; Butler et al. 2003; Marchetti et al. 2004). Apoptosis is considered the main form of β-cell death for multiple low-dose STZ with the percentage of apoptotic β–cells at high levels at day 5 and 11 post-injection (O'Brien et al. 1996). Although apoptosis is not a major reason for high dose STZ induced β-cell death, the apoptosis incidence was estimated with caspase-3 western blot and TUNEL assay. Very few apoptotic cells were detected by the TUNEL method and there were no differences between the groups in different conditions. Western blot also failed to detect apoptotic cells in pancreatic lysates of KsJ mice using an antibody to cleaved caspase-3, despite a clear positive control band in equal amounts of a control tissue, spleen. This contrasts with the published work of others showing abundant apoptosis (Aizman et al. 2010). Thus, apoptosis does not play a major role for high-dose STZ-mediated cell destruction, at least for the time points chosen. Our data suggest that *in vivo* there is simply a low frequency of β-cell apoptosis with rapid clearance or disposal. Indeed this turnover has been estimated to be from 1.7min (Kurrer et al. 1997) to 11min (Augstein et al. 1998) *in vivo* for NOD mice and BB rats two alternative models of type 1 diabetes. Alternatively, the time window chosen here may have missed the major wave of apoptosis. In addition to this, the infiltrating immune cells, rather than β-cell death could contribute to the number of apoptotic cells in and surrounding islets (Augstein et al. 1998). The present data do not support a role for GCs in β-cell apoptosis at least as a major factor underlying the difference in β-cell numbers in MIP-HSD1 mice basally or post-STZ.

STZ induced β-cell destruction is dependent on the density of GLUT2. Although GCs suppress GLUT2 expression level in β-cells (Ogawa et al. 1992), our previous results in the high fat induced diabetes model suggest glucose is, if anything, better metabolized in MIP-HSD1$^{tg/+}$ β-cell (Described in chapter 4). This implies glucose is likely efficiently taken up by GLUT2 in MIP-HSD1$^{tg/+}$ mice, and strongly argues against the case that MIP-HSD1$^{tg/+}$ β-cell sequester less STZ, as a mechanism of lower initial β-cell destruction. This notion is supported by the comparable initial high glucose levels following high dose STZ in KsJ and MIP-HSD1. GLUT2 is also expressed in hepatocytes and proximal renal tubules of kidney (Burcelin et al. 1992; Noonan et al. 2001) where STZ can transiently contribute to the hyperglycaemia.

Whilst we have not estimated liver and kidney GLUT2 expression, we predict that non-pancreatic effects for STZ are comparable in all genotypes.

In conclusion, the data showed that chronic modest elevation of 11β-HSD1 specifically in pancreatic β-cells ameliorats the hyperglycaemia induced by high and low dose STZ. Even though the mechanism by which high 11β-HSD1 mediates its effects are only partly elucidated so far, these results strongly demonstrated that β-cell proliferation and anti-inflammatory effects of 11β-HSD1 play a crucial role in protection of β-cells from STZ injury and recovery. MIP-HSD1$^{tg/tg}$ mice exhibited a less protective phenotype. This implies, as for the high fat diet induced diabetes (Described in chapter 4), that GC dose is critical to the protective effect.

Chapter 6

General Discussion

6 General discussion and future work

6.1 Major findings of the thesis

The work presented in this thesis has provided new insights into both the physiological and pathological role of the intracellular glucocorticoid regenerating enzyme 11β-HSD1 in the pancreatic β-cell.

Firstly, the enzyme was shown to be expressed in β-cells with a predominant role to amplify local glucocorticoid action. The elucidation of the major promoters in regulating 11β-HSD1, P1 and P2 indicate that it is likely to be regulated by the same factors that regulate its expression in liver and adipose tissue. This promoter pattern predicts that 11β-HSD1 will be sensitive to the changes of C/EBPs, metabolic effectors and inflammatory modulators. Indeed, the first evidence is provided that islet 11β-HSD1 is down-regulated by high glucose, insulin under physiological glucose levels and is up-regulated by free fatty acids and the proinflammatory cytokine TNFα. These data provide the first clue that factors involved in physiological regulation of obesity and/or diabetes might lead to the dysregulation of islet 11β-HSD1. Importantly, the synthetic glucocorticoid (dexamethasone) does not regulate islet 11β-HSD1 even at a supraphysiological dose, which suggests the local or systemic corticosterone is not a driver for altering islet 11β-HSD1 in diabetes.

Having shown that β-cell 11β-HSD1 is elevated in a number of obesity models, including high fat diet (a modest increase with compensation in C57BL/6J, but a reduction in C57BL/KsJ with β-cell failure) and diabetic $Lep^{db/db}$ mice (a larger increase with β-cell failure), it was reasoned that 11β-HSD1 might dynamically regulate the insulin secretory capacity of the β-cell. Given the weight of evidence to suggest that GCs negatively affect GSIS, but some of the conflicting data in the literature that GCs may also improve aspects of β-cell function (details in 1.4.3.3.4),

a transgenic model of β-cell specific 11β-HSD1 overexpression was therefore created. It was hypothesized that high level of 11β-HSD1 would amplify local GC action and suppress GSIS. On the contrary, as shown in chapter 4, modest elevation of 11β-HSD1 in the β-cells of the β-cell failure-susceptible KsJ line (MIP-HSD1$^{tg/+}$) – to the level seen in the robustly compensating C57BL/6J line – completely reversed β-cell failure. Homozygote mice (MIP-HSD1$^{tg/tg}$) with higher again 11β-HSD1 overexpression tipped the islet function over a protective threshold, leading to suppressed basal GSIS, suggesting that the discrepancies observed in the literature might be reconciled as being a result of GC dose. Despite this inverted U-shaped dose response, a hallmark of GCs action in other systems (Du et al. 2009), the MIP-HSD$^{tg/tg}$ mice retained a partial protection from HF-induced β-cell failure. This suggests that the mechanisms leading to suppression or stimulation of GSIS by GCs can be dissected, and this may offer the potential for future exploration of β-cell compensatory mechanisms. Indeed, more recent work based on a microarray comparison on MIP-HSD1$^{tg/+}$ and KsJ islets from HF fed mice suggests that protein kinase A, heat-shock and ERK signaling pathways may underlie the remarkable protection from β-cell failure in this model (Turban et al. unpublished). Of note, there was no major effect of the MIP-HSD1$^{tg/+}$ on inflammatory signaling pathways that had been proposed as the protective mechanism of corticosterone-mediated improvement of GSIS in islets (Hult et al. 2009). It is likely that both the degree of inflammatory damage in the type 2 diabetes model is lower than a type 1 diabetes environment and that the level of GC regenerated by the modest 11β-HSD1 overexpression (MIP-HSD1$^{tg/+}$) is more physiological than that observed with a clearly immuno-suppressing dose of corticosterone (200nM) used by Hult et al. Nevertheless, it was hypothesized based on the protection from type 2 diabetes that MIP-HSD1 mice might be protected from the pronounced inflammatory insult induced by STZ-induced type 1 diabetes in chapter 5. This reversed hypothesis was shown to be correct. Thus it was shown the MIP-HSD1$^{tg/+}$ and to a lesser extent MIP-HSD1$^{tg/tg}$ islets were protected from high and low dose STZ-induced β-cell damage through reduction of inflammatory cell infiltration of the innate immune response and increased infiltration pro-resolution T cells of the adaptive immune response.

6.2 GC action and 11β-HSD1 in islets: a new perspective

GCs are well known for their profound physiological effects, and are used as effective anti-inflammation medicines at high doses. However, long-term corticosterone treatment and excessive endogenous glucocorticoids, as seen in Cushing Syndrome promotes metabolic disorders which contribute to diabetes (Landy et al. 1988; Chrousos 1995; Dorn et al. 1995; Reynolds et al. 2001; Andrews et al. 2002). There is disagreement among previous studies on the predominant direct effects of GCs on islet function. This may reflect the different species, dose and timing of exposure used (Gremlich et al. 1997; Lambillotte et al. 1997; Jeong et al. 2001; Rafacho et al. 2008; Swali et al. 2008; Hult et al. 2009; Rafacho et al. 2010). Transgenic manipulation of 11β-HSD1 in the β-cell has revealed the proponents of both suppressive and stimulatory effects of GCs on islet function are likely correct; this may largely depend on the (local) dose/length of exposure level, within the context of altered demand on the pancreas; for example corticosterone will potently suppress inflammatory processes in islets (Hult et al. 2009), however, *in vivo*, any suppressive effect on GSIS might be counteracted by the increased secretory demand caused by muscle insulin resistance/hepatic glucose output. The findings reconcile the controversy in the field, with the exception of the RIP-GR model, in which increased β-cell glucocorticoid sensitivity by overexpression of GR in β-cell led to suppressed GSIS in the absence of altered systemic insulin sensitivity (Delaunay et al. 1997; Ling et al. 1998; Davani et al. 2004). This discrepancy suggests that there are distinctions between the models. For example, elevated 11β-HSD1 may metabolize other ligands relevant to insulin secretion such as 7-oxysterols (Wamil et al. 2008) that can affect GSIS by activating liver X receptor (Efanov et al. 2004). RIP-GR mice may alter the balance of the amount of GC that activates stimulatory mineralocorticoid receptors in β-cells (Koizumi and Yada 2008), whereas MIP-HSD1 mice will provide increased ligand to both. RIP-GR mice may have a detrimentally altered developmental impact on later adult islet function (Breant et al. 2006), whereas MIP-HSD1 have normal islet numbers before birth (Turban et al. unpublished). In any case, there is more evidence to suggest 11β-HSD1 is altered in

pancreatic islets in obesity and/or diabetes rather than GR (Duplomb et al. 2004; Ortsater et al. 2005), suggesting MIP-HSD1 mice are the more relevant model of physiological changes or disease. Future work will dissect out the exact molecular events underlying the different effects between GR and 11β-HSD1 in islet.

Overall, the data point to islet 11β-HSD1 as having a physiological role in compensatory GSIS in the face of insulin resistance. Indeed this will be one of the few β-cell compensatory mechanisms described in the literature, and these mechanisms are highly sought after to begin the task of intervention in β-cell failure and diabetes prevention. Currently advocated therapeutic strategies to inhibit the enzyme, whilst having clearly beneficial effects on whole body insulin sensitivity may therefore need to be assessed cautiously for β-cell-specific effects in susceptible patients (Rosenstock et al. 2010).

6.3 GC effects on β-cell development

Our transgenic mice used the insulin promoter to drive 11β-HSD1 gene expression. During mouse development insulin appears at e9.5 within the developing pancreatic bud (Teitelman et al. 1993), which suggests the MIP-HSD1 mice would express the 11β-HSD1 gene at this stage which is earlier than its normal expression time at e13 (Speirs et al. 2004). GCs exert their effects by binding to GR. GR mRNA can be detected in foetal pancreatic region at e13.5–e14, increases at e14.5-e16.5 in both islet progenitor cells and the majority of ductal cells, then falls sharply (Speirs et al. 2004). Therefore, the MIP-HSD1 mice would presumably have more GC action on developing pancreas since GR is expressed there. Previous reports strongly suggest that excessive GC exposure in the developing pancreas reduces the number and function of mature β-cells and causes permanently impaired glucose tolerance (details in 1.4.3.1). As discussed above, optical projection tomography (OPT) showed no difference in islet number between KsJ and MIP-HSD1$^{tg/+}$ at e18 and in newborns (Turban et al. unpublished) which suggest the elevation of GCs in MIP-HSD1$^{tg/+}$ mice has not affected the β-cell development, at least in the prenatal

period. Further work has to be done on MIP-HSD1$^{tg/tg}$ mice, which have an impaired second phase insulin secretory response even on normal chow diet. This might be consistent with the reports of others that excessive GCs in development reduced the number of functional β-cell (Garofano et al. 1997; Garofano et al. 1998; Nyirenda et al. 2009).

6.4 GC effects on β-cell replication and apoptosis

MIP-HSD1$^{tg/+}$ mice exhibit more small islets than KsJ (Turban et al. unpublished). As 11β-HSD1 does not affect β-cell generation and maturation during development (Turban et al. unpublished), and there is no evidence for altered proliferation and apoptosis (see chapter 5), the neonatal and weaning stages may be the critical windows during which higher 11β-HSD1 activity might drive increased β-cell proliferation and/or reduced apoptosis leading to an increased pancreatic β-cell mass (Scaglia et al. 1995). Proliferation and apoptosis rates/markers will be assessed for MIP-HSD1 mice at these time periods in future work.

6.5 Interaction between islet α-cell and β-cell

In this thesis the effects of MIP-HSD1 mice on α-cells have not been investigated. The islet α-cell secretes glucagon which elevates blood glucose through glycogenolysis and gluconeogenesis by binding to the glucagon receptor in liver. Glucagon potently stimulates β-cell inulin release to maintain glucose homeostasis (Gromada et al. 1997). The effects of GC on glucagon secretion are not clear. Prednisolone increased glucagon secretion in mice *in vitro* (Marco et al. 1976) in agreement with clinical studies (Wise et al. 1973). However, 50nM corticosterone and 11DHC reduced mouse islet glucagon secretion *in vitro* (Swali et al. 2008). Moreover, 10nM dexamethasone decreased glucagon receptor mRNA expression in rat islets (Abrahamsen and Nishimura 1995). Therefore, it will be important to investigate whether MIP-HSD1$^{tg/+}$ mice with increased islet insulin secretion, exhibit

altered glucagon paracrine profiles in future. The role of α-cell 11β-HSD1 expression (Swali et al. 2008) will also be important to examine in conjunction with changes in the β-cell.

6.6 Glucose metabolism and the insulin secretion mechanism

MIP-HSD1$^{tg/+}$ mice display a clearly compensatory secretory response to high levels of glucose stimulation after high fat feeding. This suggests an optimal concentration of GCs is a positive physiological adaptive response supporting hyperinsulinaemia. Although the mechanism is largely unknown, this may affect the factors involving in glucose metabolism and/or insulin secretion, such as NAD(P)H/NAD(P)$^+$ ratio (Rafacho et al. 2010), intracellular Ca^{2+} concentration (Rafacho et al. 2010; Sood and Ismail-Beigi 2010) and insulin secretory granule release (Rafacho et al. 2010). Moreover, factors affecting GSIS can differentially regulate β-cell 11β-HSD1 activity (see chapter 3) such as high glucose, insulin as well as palmitate and TNFα. Besides the preliminary identification of PKA (increasing insulin secretion (Gao et al. 2002))/Hsp (reducing ER stress (Hult et al. 2009))/ERK pathways (enhancing differentiation (Ehses et al. 2002)) as underlying the beneficial changes (Turban et al. unpublished), future work will build upon the impact of (modest, chronically) altered GC exposure on the secretory machinery of the β-cell including membrane potential, G-protein coupled receptor signalling (e.g. GLP-1 responsiveness, a major incretin hormone that signals increased insulin secretion and islet survival), calcium and NADPH flux. In the latter case, one might expect the increased 11β-HSD1 activity to deplete NADPH levels, at least in the ER where the co-factor is provided by H6PDH. This intriguing reversal of the co-factor shuttle might be expected to reduce GSIS, as NADPH is a proximal stimulatory factor in this process (Ivarsson et al. 2005; Ronnebaum et al. 2006). Clearly, any 11β-HSD1-mediated NADPH consumption does not show negative impact on GSIS, at least on the heterozygote MIP-HSD1$^{tg/+}$ mice.

6.7 11β-HSD1 knockout mice and implications for therapeutic 11β-HSD1 inhibitors

The global deletion of 11β-HSD1 and the utilization of 11β-HSD1 inhibitors showed protective effects in metabolic disease (see 1.5.5 and 1.5.6) in laboratory animals as well as in some clinical trials (details in 1.5.6). The prospects for therapeutic inhibition of 11β-HSD1 are therefore raised, although the effects of 11β-HSD1 in some tissues and systems have not been fully explored. Indeed modestly elevated 11β-HSD1 in β-cell (MIP-HSD1$^{tg/+}$) showed protective effects from high fat diet- and STZ-induced diabetes. Moreover, 11β-HSD1 knock out mice exhibit a mild β-cell insulin secretory defect (Turban et al. unpublished) although this is masked under normal insulin sensitivity and in particular under the dominant beneficial effects of peripheral insulin sensitization with HF diet (Morton et al. 2004). Whilst further work will be needed to confirm whether 11β-HSD1 is similarly regulated in human islets, and plays a functional role in GSIS, it must be concluded that 11β-HSD1 inhibitors at least be given with caution. Close attention may have to be paid to those individuals who may have a genetic predisposition for β-cell failure or some indication of β-cell insufficiency (e.g. risk-associated HOMA-B) (Dupuis et al. 2010). The work in this thesis therefore gives an immediately clinically relevant insight into the clinically effective dose of steroids or 11β-HSD1 inhibitors in therapeutic treatment of diabetes.

Chapter 7

References

7 References

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